

# **Ureide metabolism in response to abiotic stresses in Arabidopsis**

A Thesis Submitted to the College of Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Biology

University of Saskatchewan

By

**Solmaz Irani**

2016

## **Permission to Use**

In presenting this thesis/dissertation in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis/dissertation in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis/dissertation work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis/dissertation or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis/dissertation.

## **Disclaimer**

Reference in this thesis/dissertation to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation, or favouring by the University of Saskatchewan. The views and opinions of the author expressed herein do not state or reflect those of the University of Saskatchewan, and shall not be used for advertising or product endorsement purposes.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Head of the Department of Biology  
112 Science Place, University of Saskatchewan  
Saskatoon, Saskatchewan  
S7N 5E2 Canada

## ABSTRACT

Ureides are nitrogenous compounds derived from purine catabolism that contribute to nitrogen recycling in plants. Accumulation of some ureide compounds has been reported in a number of plants under stress conditions, suggesting their involvement in a plant response to stress. Therefore, a biochemical and molecular approach was applied to address the ureide accumulation under drought and increased light intensity stress conditions as well as NaCl and mannitol treatments in *Arabidopsis thaliana*. Ureide concentration and changes in expression of ureide metabolic genes were examined in response to these stress conditions. Additionally, an *Arabidopsis* allantoinase (*aln*) mutant with elevated accumulation of the ureide compound, allantoin, was used to investigate the impact of high levels of this compound on stress response. In the leaf tissue of adult plants allantoin accumulated in response to drought and increased light intensity. Simultaneously, transcription of urate oxidase (*UO*), involved in allantoin production, was highly up-regulated under stress. Ureides, allantoin and allantoate, also accumulated in seedlings following treatment with NaCl or mannitol. Allantoinase-negative mutants with increased levels of allantoin exhibited higher tolerance to drought and NaCl stresses and growth under high light. Lower level of reactive oxygen species (ROS) accumulated in the allantoinase-negative mutant leaves in response to drought compared to the wild-type. Higher concentrations of allantoin in the mutant elevates abiotic stress tolerance, possibly by reducing oxidative damage. These results suggest that ureide metabolism and accumulation contributes to the abiotic stress response which is regulated, at least in part, at the transcript level.

## Acknowledgements

I would like to thank my supervisor Dr. Christopher Todd for his guidance and assistance throughout my research. I found him very eager and enthusiastic in teaching and sharing his knowledge in both theoretical and practical parts of science. I particularly want to thank him for being my mentor in most of my lab work including but not limited to HPLC and cloning.

Many thanks to Drs. Peta Bonham-Smith, Carlos Carvalho and Gordon Gray for their contributions to this project. Thank you to Dr. Bonham-Smith and Dr. Carvalho for the use of their laboratory equipment and Dr. Gray for providing me access to his growth chambers. I greatly appreciate Dr. Elizabeth Weretilnyk for serving as my external examiner and Dr. Yangdou Wei for finding the time to chair my thesis defense.

I would like to acknowledge Natural Sciences and Engineering Research Council of Canada (NSERC) and other financial support throughout my studies. My Ph.D. study was supported by the Dean's scholarship from the College of Graduate Studies as well as the University Graduate Scholarship and Graduate Teaching Fellowship from the Department of Biology.

I would like to thank Dr. Ken Wilson and also the previous and current members of the Bonham-Smith, Kaminskyj and Andres labs for access to their laboratories and expertise. A special thank you to Dr. Vipen Sawhney for his advice and encouragement during my Ph.D.

Thank you to Marlynn Mierau and Dr. Guosheng Liu for their valuable help with my research and the staff of the Biology Department especially Joan Virgl, Deidre Wasyliw and Halyna Heisler for their precious aid. I am thankful to Gillian Murza, Jill Thomson, other instructors and TA's for their guidance during my work as teaching assistant.

I am very thankful of all the past and present labmates for their assistance during my research. I am also deeply grateful to my many friends in the department of Biology and University of Saskatchewan for their time and support. I cannot express how thankful I am for your companionship and friendship.

Most of all, thanks to my dear mom and sister, for their considerations, encouragement, patience and constant support.

And finally, this thesis is dedicated to the loving memory of my father, who passed away during my Ph.D. studies.

## TABLE OF CONTENTS

<b>PERMISSION TO USE</b> .....	<b>i</b>
<b>ABSTRACT</b> .....	<b>ii</b>
<b>ACKNOWLEDGMENTS</b> .....	<b>iii</b>
<b>TABLE OF CONTENTS</b> .....	<b>iv</b>
<b>LIST OF TABLES</b> .....	<b>vii</b>
<b>LIST OF FIGURES</b> .....	<b>viii</b>
<b>LIST OF ABBREVIATIONS</b> .....	<b>xiv</b>
<b>CHAPTER 1. INTRODUCTION</b> .....	<b>1</b>
1.1. Plants and abiotic stress.....	1
1.1.1. Abiotic stress and hormones .....	2
1.1.2. Abiotic stress and ROS .....	5
1.1.3. Abiotic stress and accumulation of specific compounds in plants .....	8
1.1.4. Abiotic stress and organelles .....	10
1.2. Increased light intensity .....	12
1.3. Osmotic stress .....	14
1.4. Drought .....	15
1.5. Salinity stress .....	17
1.6. Purine-derived ureide metabolism.....	19
1.6.1. Ureide transporters .....	20
1.7. Historical overview of ureides in plants .....	22
1.7.1. 1930s-1970s .....	22
1.7.2. 1970- 1995: Ureides in tropical legumes .....	23
1.7.3. 1995- 2005: Ureides in tropical legumes under water stress .....	24
1.7.4. After 2005: Ureides and nitrogen fixation under abiotic stress .....	24
1.7.5. Ureides in non-legume plants under abiotic stress .....	25
1.8. Putative functions of ureides during plant stress .....	27
1.9. Research questions .....	30
<b>CHAPTER 2. METHODS AND MATERIALS</b> .....	<b>32</b>
2.1. Plant material and growth conditions .....	32
2.2. T-DNA insertion mutant lines.....	33
2.2.1. PCR-based screen .....	33
2.2.2. DNA extraction.....	35
2.2.3. PCR conditions .....	35

2.2.4. Back cross .....	36
2.3. Abiotic stress conditions .....	37
2.3.1. Drought .....	37
2.3.2. Light treatments .....	39
2.3.3. NaCl and mannitol .....	39
2.3.4. NaCl tolerance test.....	39
2.3.5. NaCl and exogenous allantoin tolerance test .....	40
2.4. Quantification of chlorophyll and anthocyanin .....	40
2.5. Photochemical efficiency of PSII .....	41
2.6. Staining and quantification of O <sub>2</sub> <sup>-</sup> and H <sub>2</sub> O <sub>2</sub> .....	41
2.7. Sample preparation for analysis by HPLC.....	42
2.7.1. HPLC parameters.....	42
2.8. Gene expression analysis .....	43
2.8.1. RNA extraction and cDNA synthesis .....	43
2.8.2. Reverse transcriptase PCR.....	44
2.8.3. Quantitative reverse transcriptase PCR.....	44
2.9. Statistical analysis .....	46
<b>CHAPTER 3. RESULTS.....</b>	<b>47</b>
3.1. T-DNA insertion mutants .....	47
3.1.1. Characterization of homozygous mutants.....	47
3.1.2. Gene expression analysis .....	47
3.1.3. Preliminary phenotypic tests.....	53
3.2. Drought stress .....	57
3.2.1. Ureide concentration in wild-type leaves.....	57
3.2.2. Expression analysis of ureide genes in wild-type leaves .....	57
3.2.3. Soil gravimetric water content .....	62
3.2.4. Drought stress tolerance of <i>aln-3</i> plants compared to wild-type .....	62
3.2.5. Chlorophyll content and the maximum efficiency of PSII .....	67
3.2.6. Levels of O <sub>2</sub> <sup>-</sup> and H <sub>2</sub> O <sub>2</sub> .....	67
3.3. Increased light light intensity .....	70
3.3.1. Ureide concentration in wild-type leaves by light treatments.....	70
3.3.2. Expression analysis of ureide genes by RT-PCR.....	70
3.3.3. Tolerance of <i>aln-3</i> plants under increased light intensity compared to wild-type .....	73
3.3.4. Chlorophyll and anthocyanin content and the maximum efficiency of PSII .....	73
3.4. NaCl and mannitol treatments.....	78

3.4.1. Ureide concentration in wild-type seedlings.....	78
3.4.2. Expression analysis of ureide genes in wild-type seedlings .....	78
3.5. NaCl tolerance of the <i>aln-3</i> seedlings compared to the wild-type .....	82
3.5.1. Col-0 and <i>aln-3</i> seedling tolerance to NaCl .....	82
3.5.2. Seedlings stress tolerance when transferred to NaCl media .....	82
3.6. Effect of exogenous allantoin on the tolerance of wild-type seedlings under NaCl stress .....	87
3.6.1. Effect of exogenous allantoin when seeds are germinated on NaCl .....	82
3.6.2. Effect of exogenous allantoin when seedlings are transferred to NaCl media.....	92
<b>CHAPTER 4. DISCUSSION.....</b>	<b>97</b>
4.1. Accumulation of allantoin in Arabidopsis leaves under drought stress .....	97
4.2. Accumulation of allantoin in Arabidopsis leaves under increased light intensity .....	98
4.3. Enhanced levels of allantoin and allantoate in seedlings by NaCl and mannitol treatments .....	100
4.4. Expression of ureide metabolism genes under abiotic stress conditions .....	101
4.5. Arabidopsis ureide mutants and their performance under abiotic stress.....	103
4.6. Effect of exogenous allantoin on increasing the tolerance of seedlings to NaCl stress .....	105
4.7. Allantoin .....	107
4.7.1. The function of allantoin under abiotic stress.....	108
4.8. Principle questions addressed in this thesis and anticipated significance of research .....	111
4.9. Remaining questions .....	115
<b>5. APPENDIX A. Cloning .....</b>	<b>117</b>
<b>6. APPENDIX B. Supplementary tables.....</b>	<b>137</b>
<b>7. APPENDIX C. Supplementary figures.....</b>	<b>144</b>
<b>8. REFERENCES.....</b>	<b>155</b>

## LIST OF TABLES

Table 1.1. High ureide concentration in non-legume plants under abiotic stress	28
Table A1. List of primers used to amplify the promoter or coding sequence regions of ureide genes	122
Table A2. PCR conditions used for amplification of each sample	124
Table A3. Restriction enzymes used for double-digestion of <i>XDHI</i> promoter and <i>UO</i> coding sequence	126
Table B1. T-DNA insertion lines ordered from ABRC	137
Table B2. Sequence of primers designed with T-DNA primer design program and used to screen T-DNA insertion lines	138
Table B3. List of primers used to detect the effect of T-DNA insertion on the expression of impaired gene by RT-PCR	139
Table B4. List of primers used for gene expression analysis under abiotic stress conditions using RT-PCR	140
Table B5. List of primers used for qRT-PCR	141
Table B6. Homozygous T-DNA insertion lines and the effect of insertion on the expression of the mutated gene detected by RT-PCR	142
Table B7. Chlorophyll level (A), $F_v/F_m$ ratio (B) and anthocyanin level (C) of Col-0 and <i>aln-3</i> leaves on day 0, 7, 14 and 21 after light treatments	143



## LIST OF FIGURES

Figure 1.1. The main steps of ureide biosynthesis and catabolism in Arabidopsis in cytosol, peroxisome and endoplasmic reticulum	21
Figure 2.1. PCR-based screening of T-DNA insertion mutants	34
Figure 2.2. Schematic procedures of backcross of <i>aln-3</i> against the Col-0	38
Figure 3.1. The screening procedures of T-DNA insertion mutant lines	48
Figure 3.2. An example image representing the genotyping of a T-DNA insertion mutant Line	49
Figure 3.3. RT-PCR analysis to detect the effect of T-DNA insertion in the gene on gene expression of the four homozygous mutants	50
Figure 3.4. Diagram of T-DNA insertion in the <i>XDHI</i> , <i>ALN</i> , <i>AAH</i> and <i>UAH</i> genes in the mutants	51
Figure 3.5. Ureide metabolism genes with T-DNA insertions resulting loss of transcript	52
Figure 3.6. Plant phenotypes under increased light condition	55
Figure 3.7. Plant phenotypes under moderate light condition	56
Figure 3.8. Allantoin concentration in Col-0 leaves in response to drought and well-watered conditions	59
Figure 3.9. RT-PCR analysis of a number of ureide metabolic genes under drought stress	60

Figure 3.10. qRT-PCR analysis of <i>UO</i> and <i>ALN</i> transcripts in Col-0 leaves under drought and well-watered conditions	61
Figure 3.11. Soil gravimetric water content (SGWC) during withholding of water	63
Figure 3.12. Phenotypes of Col-0 and <i>aln-3</i> plants following withholding of water and re-watering	64
Figure 3.13. Biomass of Col-0 and <i>aln-3</i> plants following withholding and well-watered conditions	65
Figure 3.14. Allantoin level in <i>aln-3</i> leaves in response to drought	66
Figure 3.15. Chlorophyll content and the maximum efficiency of PSII	68
Figure 3.16. Staining and quantification of $O_2^-$ and $H_2O_2$	69
Figure 3.17. Allantoin accumulation in Col-0 leaves in response to increased light intensity	71
Figure 3.18. RT-PCR analysis of ureide metabolism genes under increased light Condition	72
Figure 3.19. The effect of increased light condition on the phenotype of Col-0 and <i>aln-3</i> plants	74
Figure 3.20. The effect of moderate light condition on the phenotype pf Col-0 and the <i>aln-3</i> plants	75
Figure 3.21. Allantoin and uric acid contents in <i>aln-3</i> leaves under increased light conditions	76

Figure 3.22. Chlorophyll and anthocyanin levels and the maximum efficiency of PSII ( $F_v/F_m$ ratio) on day 14 after moderate and increased light treatments	77
Figure 3.23. Phenotype of Col-0 seedlings before and 4 days after NaCl and mannitol treatments	79
Figure 3.24. Ureide concentration in response to NaCl and mannitol treatments	80
Figure 3.25. RT-PCR analysis of ureide genes, 4 days after NaCl and treatments	81
Figure 3.26. Phenotypes of Col-0 and <i>aln-3</i> seedlings following germination under NaCl stress	83
Figure 3.27. Percentage of seedlings with true leaf and the root length of seedlings germinated on NaCl media for 7 days	84
Figure 3.28. Phenotype of seedlings before and after shift to media with 100 mM NaCl and without NaCl	85
Figure 3.29. Fresh weight and root length of 2-week-old seedlings under 0 and 100 mM NaCl	86
Figure 3.30. Experimental design to detect the effect of exogenous allantoin on Col-0 when germinated on NaCl	88
Figure 3.31. Fresh weight of Col-0 seedlings after 7 and 10 days of germination on six different media	89
Figure 3.32. Root length of Col-0 seedlings, 10 days after germination on six different media	90

Figure 3.33. Phenotype of 10-day-old Col-0 seedlings germinated under three different NaCl media	91
Figure 3.34. Experimental design to detect the effect of exogenous allantoin on Col-0 seedlings' growth on NaCl media	93
Figure 3.35. Fresh weight of 2-week-old Col-0 seedlings grown on six different media	94
Figure 3.36. Root length of 2-week-old Col-0 seedlings grown on six different media	95
Figure 3.37. Phenotype of 2-week-old Col-0 seedlings grown on four different media	96
Figure 4.1. Proposed model depicting the effect of abiotic stress on ureide metabolism	113
Figure 4.2. Proposed model for the possible role(s) of allantoin in Arabidopsis under abiotic stress	114
Figure A1. pCAMBIA1303 vector structure	121
Figure A2. PCR amplification of the promoters and coding sequence of four ureide genes using primers with restriction enzyme sites and extra overhang nucleotides	129
Figure A3. EcoRI restriction enzyme analysis of ureide genes cloned into the TOPO plasmid vector	130
Figure A4. Colony-PCR results of positive <i>E. coli</i> and <i>A. tumefaciens</i> colonies	132

Figure A5. Identification of transformant seedlings on 25 mg L <sup>-1</sup> hygromycin selection plates	133
Figure A6. Potential transformant seedlings after 10 days growth on fresh hygromycin selection plate	134
Figure C1. HPLC standard calibration curves for allantoin, uric acid and allantoate	144
Figure C2. HPLC separation of ureide	145
Figure C3. Control RTPCR analysis of four homozygous mutants without expression of the mutant genes	146
Figure C4. Phenotype of Col-0 and mutant seedlings when seeds were germinated on MS control	147
Figure C5. Phenotype of Col-0 and mutant seedlings when seeds were germinated on 150 mM NaCl	148
Figure C6. Uric acid level in Col-0 leaves in response to drought and well-watered conditions	149
Figure C7. RT-PCR analysis of ureide metabolic genes under well-watered condition	150
Figure C8. Allantoin content in <i>aln-3</i> in response to drought and control conditions	151
Figure C9. RT-PCR analysis of ureide metabolism genes in Col-0 leaves, 10 days after moderate and high light treatments	152
Figure C10. Percentage of germinated Col-0 and <i>aln-3</i> seeds, 3 days after transfer to	

germination-permissive conditions 153

Figure C11. Percentage of germinated Col-0 seeds, 3 and 6 days after transfer to  
germination-permissive conditions 154

## LIST OF ABBREVIATIONS

ABA	abscisic acid
ABRC	Arabidopsis Biological Resource Center
Abs	absorption
AAH	allantoate amidohydrolase
ACT	actin
ALN	allantoinase
ALN	allantoin
ALNS	allantoin synthase
ANOVA	analysis of variance
APX	ascorbate peroxidase
At	<i>Arabidopsis thaliana</i>
ATP	adenosine triphosphate
BC	backcross
BLAST	basic local alignment search tool
BP	T-DNA border primer
bp	base pair
CaMV	cauliflower mosaic virus
CAT	catalase
CDS	coding sequence
Chl	chlorophyll
cDNA	complementary DNA
CSD	Cu/Zn superoxide dismutase
Col.0	Columbia
DAB	diaminobenzidine
ddH <sub>2</sub> O	deionized distilled water
DMSO	dimethyl sulfoxide
DW	dry weight
EC	enzyme commission number
EDTA	ethylene diamine tetra acetic acid

EtBr	3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide
ER	endoplasmic reticulum
F	filial generation
$F_m$	maximal fluorescence in the dark-adapted state
$F_o$	background fluorescence in the dark-adapted state
$F_v$	variable fluorescence ( $F_m - F_o$ )
$F_v/F_m$	photochemical efficiency of PSII ( $F_m - F_o$ )/ $F_m$
FW	fresh weight
$g$	gravitational force
GFP	green florescent protein
GUS	beta glucuronidase
<i>Gm</i>	<i>Glycine max</i>
GR	glutathione reductase
h	hour
5-HIU	5-hydroxyisourate
HClO <sub>4</sub>	perchloric acid
His	histidine
HPLC	high-performance liquid chromatography
HM	homozygous
HSD	honest significant difference
HT	heterozygous
LB	Luria-Bertani medium
Lpe	leaf permease
LP	left primer
MS	Murashige-Skoog medium
mRNA	messenger RNA
N	nucleotide
n	number of independent experiments
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NCBI	National Center for Biotechnology Information



NCED	9-cis-epoxycarotenoid dioxygenase
O <sub>2</sub> <sup>-</sup>	superoxide anion
OHCU	2-oxo-4-hydroxy-4-carboxy-5-ureido-imidazoline
P	promoter
PCR	polymerase chain reaction
PPDF	Photosynthetic photon flux density
Pfu	<i>Pyrococcus furiosus</i> DNA polymerase
Pro	proline
<i>P</i> -value	( <i>P</i> : probability)
P5CS	pyrroline-5-carboxylate synthase
PSI	photosystem I
PSII	photosystem II
PUP	purine permease1
<i>Pv</i>	<i>Phaseolus vulgaris</i>
qRT-PCR	quantitative reverse transcriptase PCR
RD	dehydration-responsive genes
RNAi	RNA interference
ROS	reactive oxygen species
RP	right primer
RT-PCR	reverse transcriptase PCR
SAIL	Syngenta Arabidopsis Insertion library
SALK	Institute for biological studies
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SGWC	soil gravimetric water content
SOD	superoxide dismutase
T-DNA	transfer DNA
TSS	transcription start site
UO	uricase
UPS	ureide permease1
UTR	untranslated region

v	volume
w	weight
WT	wild-type
XDH	xanthine dehydrogenase

## CHAPTER 1. INTRODUCTION<sup>1</sup>

### 1.1. Plants and abiotic stress

All living cells are affected by their environment during growth and development. Obviously for plants this effect is more challenging since they are sessile organisms. Environmental or abiotic stresses such as low temperature, salt, drought, heat, oxidative stress and heavy metal toxicity are the most common reason for crop failure worldwide. Growth and productivity of plants are frequently affected by these environmental conditions (Mahajan and Tuteja, 2005). Abiotic stresses threaten the sustainability of farming and investments in the agriculture industry by influencing both crop yield and quality (Amudha and Balasubramani, 2011). Moreover, agricultural output needs to increase to meet the world food demand, especially considering the growing population of the Earth during the next decades (Tilman *et al.*, 2002). Therefore, understanding the abiotic stress responses and tolerance mechanisms in plants is important for both plant biologists and agronomists (Qin *et al.*, 2011).

Plants have adjusted various biochemical and molecular mechanisms to counteract the adverse effects of different abiotic stresses (Molassiotis and Fotopoulos, 2011). Due to the complexity of stress response and tolerance, understanding the mechanisms involved in plant responses has always been challenging (Qin *et al.*, 2011). However, emerging new tools and approaches in various research areas including molecular biology, plant physiology and cell biology, have considerably enhanced our knowledge (Amudha and Balasubramani, 2011). Availability of genome information and mutant lines (e.g., T-DNA insertion lines), whole-genome tiling arrays and progress in the research areas such as functional small RNAs (siRNA and miRNA) have considerably facilitated the assessment of a plant's response to abiotic stress (Hirayama and Shinozaki, 2010).

The key strategy for plants to perceive an abiotic stress is to use a network of signals controlled by various chemical molecules and phytohormones (Tuteja, 2007). Following the chemical signals, expression of numerous genes and signaling factors involved in the different response pathways is altered (Tuteja and Sopory, 2008).

<sup>1</sup> Parts of this dissertation was reprinted from:

Irani S and CD Todd (2016) Ureide metabolism under abiotic stress in *Arabidopsis thaliana*. J Plant Physiol. 199:87-95. and are presented here in accordance with the publisher's statement of copyright.

A comprehensive molecular reprogramming under stress occurs through the up or down regulation of a wide range of stress responsive genes (Seki *et al.*, 2002; Thomashow, 2010; Liu *et al.*, 2014). Based on the function of the proteins that these genes encode, they are classified into regulatory or functional genes. For example, regulatory genes include transcription factors, protein kinases and phospholipases. On the other hand, functional genes play vital roles in protective mechanisms such as cell membrane stability, macromolecular structure maintenance and reactive oxygen species (ROS) scavenging (Shinozaki and Yamaguchi-Shinozaki, 2007).

Photosynthesis, as the key physiological function of plants, has a direct effect on plant growth. However, photosynthesis is highly sensitive to abiotic stress conditions (Chaves *et al.*, 2009; Lawlor and Tezara, 2009; Pinheiro and Chaves, 2011). Environmental stresses can suppress photosynthesis; for example, by affecting photosynthetic pigments, thylakoid membrane, soluble proteins, the electron transport chain, CO<sub>2</sub> fixation and photorespiration (Chaves *et al.*, 2009; Ashraf and Harris, 2013; Nouri *et al.*, 2015). Several studies in different plant species reported the reduction of photosynthetic pigment content, chlorophyll and carotenoids, under various abiotic stresses due to the impairment of pigment biosynthesis or increase in their degradation rate. Additionally, it has been shown that abiotic stress conditions such as drought, salt and heat reduce the activity of various photosynthetic enzymes in several plant species (Ashraf and Harris, 2013). On the other hand, environmental stresses can decrease a plants' photosynthesis rate by affecting plant water status and stomatal closure. The change in plant water status has been proposed to be one of the common effects of many abiotic stresses (Bohnert *et al.*, 1995). Water deficit in plant tissues occurs following the imbalance between water uptake by roots and leaf transpiration (Aroca *et al.*, 2012). Stomatal closure inhibits water transpiration and also decreases the availability of CO<sub>2</sub> through restriction of diffusion from the stomata and the mesophyll (Chaves *et al.*, 2009).

Plant responses to abiotic stress include a complex of molecular, physiological and biochemical processes. These responses help the plant to tolerate the stress, or in the ideal case, lead to stress adaptation. Understanding a plant's stress response mechanisms to perceive and transmit the environmental signals and adoptive responses has been critical for plant biologists and agronomists (Krasensky and Jonak, 2012). This knowledge is essential for the development of breeding strategies, stress tolerant transgenic plants, and selection of stress tolerant crop cultivars with high yield (Ahmad and Prasad, 2012). In the subsequent sections of this chapter, some of the primary responses of plants to abiotic stress are highlighted.

### 1.1.1. Abiotic stress and hormones

Phytohormones play important regulatory functions in the processes of plant growth and development under abiotic stress conditions. Some of the major plant hormones are abscisic acid (ABA), auxin, ethylene, jasmonic acid, cytokinin, and brassinosteroids, their biosynthesis and signaling system have been studied (Singh and Jwa, 2013). The interaction of these hormones is a primary determinant in a plant's response to environmental changes by transition of source/sink (e.g. by regulation of carbon partitioning between individual organs), distribution of nutrients and the control of growth and development (Peleg and Blumwald, 2011). Plants generally respond to abiotic stress by modulating gene expression through the signaling processes mediated by phytohormones (Kohli *et al.*, 2013).

ABA, which is often called the plant stress hormone, is the main and most common phytohormone that is produced in response to various abiotic stress conditions. ABA plays important roles in induction of seed dormancy, reduced leaf expansion and deeper root growth in response to stress conditions (Wilkinson *et al.*, 2012). Also, promoting stomatal closure to prevent water loss is one of the main function of ABA (Mahajan and Tuteja, 2005). ABA is involved in signaling the stressful conditions of the root environment to shoots, leading to reduction of transpiration activity of the leaves by closing stomata and reducing leaf expansion (Wilkinson *et al.*, 2012). It is known that ABA suppresses cell differentiation of columella stem cells and division of meristematic cells in response to environmental stresses such as drought and high salinity. This strategy results in elongation of the primary root (Hong *et al.*, 2013).

ABA application was shown to regulate the expression pattern of stress genes in the same way as they are regulated under cold, drought and salt stresses. The resemblance between the responses to these abiotic stresses is based on their similar effect on cell desiccation and osmotic imbalance. Overall, the stress response of these stresses coordinates the action of many genes, which may cross talk with each other. The products of stress response genes also contribute to generation of regulatory molecules including ABA. Regulatory molecules like ABA can initiate the second round of signaling in response to stress (Leung and Giraudat, 1998; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein *et al.*, 2002). It is suggested that the signaling through long distance (e.g. root to shoot) in stressed plants is likely to be controlled mainly by ABA and ROS (Lake *et al.*, 2002; Mahajan and Tuteja, 2005).

Although ABA is the main stress responsive hormone, a number of studies have suggested roles for other hormones such as auxin, brassinosteroids and cytokinin under adverse environmental conditions (Peleg and Blumwald, 2011). Exogenous application of brassinosteroids has been reported to induce the expression of some stress response genes. As a result, the levels of antioxidant enzymes and osmoprotectants were increased in cells (Divi and Krishna, 2009).

Plant roots probably sense and respond to soil moisture through the auxin response pathway (Kaneyasu *et al.*, 2007). Xu *et al.* (2013) reported that under moderate water deficit in rice and Arabidopsis, ABA maintains the growth of primary roots by regulating auxin transport. A number of studies on auxin signaling mutants (Wang *et al.*, 2009; Zolla *et al.*, 2010) and salt sensitive mutants (Zhao *et al.*, 2011) indicated that both auxin influx and signaling are important for development of lateral roots in response to salinity stress.

A higher tolerance of plants to stress was shown to be parallel with enhanced levels of cytokinins in xylem sap (Borrell *et al.*, 2000). In fact, cytokinin probably plays dual positive and negative roles under abiotic stress conditions (Zwack and Rashotte, 2015). Some studies have found a lower content of cytokinin in response to stress (Itai *et al.*, 1973; Walker and Dumbroff, 1981; Kudoyarova *et al.*, 2007) whereas other investigations have found enhanced levels of cytokinin especially under severe stress (Pospisilova *et al.*, 2005; Alvarez *et al.*, 2008). In addition, it has been known for a long time that elevated amounts of cytokinin negatively effect stomatal sensitivity to ABA (Radin *et al.*, 1982).

Higher amounts of ethylene under stress can prevent root growth and development and decrease the expansion of shoot and leaf (Sharp, 2002; Pierik *et al.*, 2006). Moreover, ethylene induces stress related leaf senescence and abscission (Abeles *et al.*, 1992). Ethylene reduces photosynthesis (Rajal and Peltonen-Sainio, 2001) and grain filling rates and ultimately decreases the direct yield (Hays *et al.*, 2007). Thus, inhibition of ethylene production under abiotic stress is more desirable agriculturally (Wilkinson *et al.*, 2010).

Jasmonates are involved in seed germination, fertilization, flowering and senescence (Feussner and Wasternack, 2002) and elevated amounts of this hormone have been reported under drought and salinity in rice (Moons *et al.*, 1997; Tani *et al.*, 2008). Du *et al.* (2013) reported that jasmonate concentration was increased significantly in response to drought and cold stresses in rice, but it was decreased under heat stress. This demonstrated that jasmonate signaling might differentially regulate the plant response to various abiotic stresses (Du *et al.*, 2013).

### 1.1.2. Abiotic stress and ROS

Formation of ROS in plants is an inevitable result of aerobic respiration. It is known that ROS are generated continuously in the chloroplast during photosynthesis through partial reduction of oxygen molecules or by shifting energy to these molecules (Gill and Tuteja, 2010a). Therefore, ROS are produced in cells at constant levels under normal metabolism. It has been suggested that ROS might contribute to plant development by playing a role in regulation of cell growth (Gapper and Dolan, 2006). For instance, Rodriguez *et al.* (2002) reported that during leaf expansion, ROS accumulates in the local extension zone and prevention of ROS formation inhibits leaf growth. However, most abiotic stress conditions increase the production of ROS and at the same time disrupt their elimination (Karuppanapandian *et al.*, 2011). Enhanced levels of ROS can cause oxidative damage to cells structure and ultimately cause cell death (Gill and Tuteja, 2010a). Oxidative stress is the term used to describe the excess levels of ROS in cells which mainly occurs under abiotic or biotic stress conditions (Sharma *et al.*, 2012). Oxidative stress can damage cells through lipid peroxidation, protein oxidation and DNA damage (Gill and Tuteja, 2010a). Lipid peroxidation can produce several lipid-derived radicals including lipid alkoxyl radicals and lipid epoxides (Sharma *et al.*, 2012). Consequently, peroxidation of lipids can lead to reduction of membrane fluidity, elevation of membrane leakiness, damage to membrane proteins as well as inactivation of ion channels and receptors (Gill and Tuteja, 2010a). Higher plants cope with the accumulation of ROS through various enzymatic and non-enzymatic antioxidants that quench the reactive molecules (Shao *et al.*, 2008). Some examples of plant antioxidant enzymes are superoxide dismutase, catalase, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase. Non-enzymatic ROS scavengers are low molecular antioxidants such as ascorbic acid, glutathione and carotenoids (Michalak, 2006).

In photosynthetic tissue, the main source of ROS generation is the chloroplast. Although mitochondria are the major location of ROS production in the other tissues, their contribution is not comparable to the chloroplast in photosynthetic tissue (Jaspers and Kangasjarvi, 2010). Peroxisomes (Foyer and Noctor, 2009), the endoplasmic reticulum, and cell walls (Jaspers and Kangasjarvi, 2010) can also be sources of ROS generation. Local antioxidant protection is important since most ROS forms (with the exception of H<sub>2</sub>O<sub>2</sub>) cannot pass through membrane aquaporins or travel long distances inside the cell (Sharma *et al.*, 2012). However, when local antioxidants in a cell cannot eliminate excess ROS, H<sub>2</sub>O<sub>2</sub> forms and penetrates cellular membranes

(through aquaporin channels) and diffuses into the other compartments within the cell (Sharma *et al.*, 2012).

The most common ROS with ability to cause oxidative damage are superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $\bullet OH$ ), perhydroxyl radical ( $HO_2\bullet$ ), alkoxy radicals ( $RO\bullet$ ) and peroxy radicals ( $ROO\bullet$ ) (Bhattacharjee, 2005). Of these,  $H_2O_2$  and  $O_2^-$  are considered to play important roles under abiotic stress (Halliwell, 2006; Gill and Tuteja, 2010, Sharma *et al.*, 2012).

Hydrogen peroxide ( $H_2O_2$ ) at low levels triggers stress tolerance and has been found to regulate photorespiration, photosynthesis, growth and development. However, high concentrations of  $H_2O_2$  oxidizes the thiol groups of some enzymes and induces programmed cell death (Gill and Tuteja, 2010).  $H_2O_2$  has the longest half-life among ROS and this small molecule can traverse between cellular compartments. As a result,  $H_2O_2$  can be a signaling molecule that is particularly important in plant stress acclimation, stomatal function, antioxidant defense, regulation of cell cycle and photosynthesis under stress (Petrov and Van Breusegem, 2012).  $H_2O_2$  levels are regulated by antioxidant systems consisting of enzymes that include catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and non-enzymatic antioxidants like glutathione, ascorbate and flavonoids (Hossain *et al.*, 2015).

Both CAT and APX antioxidants have high specificity for  $H_2O_2$  (Mhamdi *et al.*, 2010). There are three *CAT* genes in the Arabidopsis genome: *CAT1*, *CAT2*, and *CAT3* (Frugoli *et al.*, 1996). Pollen and seeds are the main locations of expression of the *CAT1* gene while *CAT2* is mainly expressed in photosynthetic tissue and *CAT3* in vascular tissue. The presence of CAT activity has been reported in a number of plant cellular compartments including peroxisome, cytosol, mitochondria and chloroplast (Mhamdi *et al.*, 2010). A number of APX isoforms have been identified in plants. For example, in Arabidopsis, APX1, APX2 and APX6 have been localized in the cytosol whereas APX3, APX4 and APX5 have been found on the microsome membrane. Moreover, there are other APX isozymes that are located in the chloroplast and peroxisome (Dabrowska *et al.*, 2007). Induced expression of a number of isoforms of APX has been found in different plants under abiotic stress conditions (Shigeoka *et al.*, 2002; Gest *et al.*, 2013). Increased expression of *APX1* under several oxidative stresses has been reported in Arabidopsis (Shigeoka *et al.*, 2002).



Superoxide ( $O_2^-$ ) is highly reactive and is generated by photosystem I within the thylakoid membrane. Photosystem I (PSI) is a multi-subunit protein complex that uses light to mediate electron transfer from a protein (plastocyanin) in the lumenal side of the thylakoid membrane to a protein (ferredoxin) in the stromal side of the membrane (Fromme *et al.*, 2001). Ferredoxin, the strong reductant electron acceptor of PSI, can reduce molecular oxygen to superoxide (Foyer *et al.*, 1994). Puntarulo *et al.*, (1988) suggested that 1-2 percent of oxygen molecules in plants are converted to  $O_2^-$ . In order to prevent damage to biological membranes,  $O_2^-$  formed at PSI can be eliminated by antioxidant enzymes. In fact, non-toxic levels of  $O_2^-$  may contribute to control root elongation and cell expansion in plants (Puntarulo *et al.*, 1988). Foreman *et al.* (2003) showed that root hair defective mutants (deficient in cell expansion) had lower levels of ROS such as  $O_2^-$  and had shorter roots compared to wild-type Arabidopsis. However,  $O_2^-$  in elevated levels is capable of oxidation of chloroplast molecules (e.g. chlorophylls) and lipids as well as the production of several other ROS such as hydroxyl radical and singlet oxygen (Pintó-Marijuan and Munné-Bosch, 2014). Concentration of  $O_2^-$  is regulated by both non-enzyme and enzymatic antioxidants, similarly to  $H_2O_2$ . The key antioxidant enzyme involved in scavenging  $O_2^-$  is superoxide dismutase (SOD) (Halliwell, 2006).

In plants, SOD is distinguished as three types based on the metal cofactor: FeSOD (iron cofactor), MnSOD (manganese cofactor) and Cu/ZnSOD (copper and zinc as cofactors) (Pilon *et al.*, 2011). MnSOD is found in the matrix of the mitochondria (Perry *et al.*, 2010). Although, FeSOD is frequently reported in the plastids (Bowler *et al.*, 1994; Kliebenstein *et al.*, 1998), some FeSOD isoforms are reported to be active in cytosolic locations (Myouga *et al.*, 2008; Armbruster *et al.*, 2009). Cu/ZnSOD is found in the peroxisomes and plastids (Ogawa *et al.*, 1995; Kliebenstein *et al.*, 1998). Expression of superoxide dismutase genes, especially for Cu/ZnSOD and FeSOD, has been reported to be affected by different stress treatments. In most cases, these treatments led to the upregulation of expression of superoxide dismutase genes, mainly Cu/ZnSOD (Pilon *et al.*, 2011).

### **1.1.3. Abiotic stress and accumulation of specific compounds in plants**

Biosynthesis and accumulation of specialized metabolites is a plant mechanism to acclimate to unfavorable environmental conditions. Most of the specialized metabolites synthesized under abiotic stresses show an antioxidative effect *in vitro*. However, in most cases,

the function of these metabolites *in vivo* has not been studied (Nakabayashi and Saito, 2015). Glucosinolates (Natella *et al.*, 2014), saponins (Okubo and Yoshiki, 2000; Vickers *et al.*, 2009), phenolamides (Velikova *et al.*, 2007), phenylpropanoids (Chang *et al.*, 2007; Shahidi and Chandrasekara, 2010) and flavonoids (Agati *et al.*, 2012) are some of the metabolites that exhibit *in vitro* antioxidant activity and it is suggested that they might be *in vivo* antioxidants. Accumulation of low molecular weight antioxidants such as ascorbic acid, glutathione,  $\alpha$ -tocopherols, proline (Smirnoff and Cumbes, 1989; Gill and Tuteja, 2010b; Signorelli *et al.*, 2014), sugars (Nishizawa *et al.*, 2008) and carotenoids (Havaux, 2014) has been frequently reported under stress. Aside from metabolites with antioxidative activity, a wide range of osmolytes accumulate in plant cells in response to abiotic stress: glucose and sucrose, sugar alcohols such as sorbitol, mannitol and glycerol, betaines such as glycine betaine, amino acids such as proline and pipecolic acid (a non-protein amino acid), methylated proline-related compounds such as proline betaine and hydroxyproline betaine, are some of the osmo-protective compounds (Rhodes *et al.*, 2002 ; Ashraf and Foolad, 2007). In addition to osmotic adjustment, a number of osmolytes have osmoprotection functions to help cells in response to osmotic stress for example by reducing oxidative damage and protecting sub-cellular structures under stress. An enhanced concentration of osmoprotectants in cells is correlated with increased stress tolerance probably through detoxification of ROS and protection of enzyme structure (Szabados *et al.*, 2011). For instance, the osmolyte proline might also function as a ROS scavenger, molecular chaperone or redox buffer under stress (Ashraf and Foolad, 2007; Verbruggen and Hermans, 2008). Another main osmolyte, glycine betaine, was also suggested to have other key roles such as modulation of ROS scavenging enzymes (Chen and Murata, 2011). However, it has not been reported to have a direct ROS scavenging activity (Deinlein *et al.*, 2014). It has been reported that exogenous application of these two osmolytes (glycine betaine and proline) improved the yield of some crops under abiotic stress conditions (Ashraf and Foolad, 2007). Also, exogenous application of proline and glycine betaine has been shown to increase the expression of genes that are involved in antioxidant defense and regulatory mechanisms in Arabidopsis (Oono *et al.*, 2003; Einset *et al.*, 2007). The osmoprotectant compounds that accumulate in stressed plants can serve different functions such as preserving the integrity of the plasma membrane, protecting enzyme or protein structure, stabilizing the photosystem II complex and quenching (ROS). Furthermore, they can act as chelating agents,

signaling molecules and low molecular weight chaperones (Chen and Murata, 2008; Valluru and Van den Ende, 2008; Szabados and Savouré, 2010; Alcázar *et al.*, 2010).

Previously it was thought that metabolites accumulated under stress probably had no essential role in a plant since they usually were found to accumulate in plants in response to stress. Recently, some of these metabolites were reported to have other major functions in plant growth and development (Ramakrishna and Ravishankar, 2011). For example polyamines were reported to increase in plants under abiotic stress conditions and it has been suggested that they play a role in modulating the antioxidant system (Liu *et al.*, 2015). In addition, under unfavorable conditions, polyamines may be involved in maintaining membrane stability (Liu *et al.*, 2007; Tiburcio *et al.*, 2014). Biologically, polyamines have the ability to bind to anionic macromolecules like proteins and nucleic acids and therefore to function in the regulation of transcription and translation (Bachrach, 2010; Gill and Tuteja, 2010b; Igarashi and Kashiwagi, 2010; Tiburcio *et al.*, 2014). Another example is proline; it is usually considered a metabolite with function under stress conditions. Increasing number of studies suggest that proline has other biological roles and it is a signaling molecule involved in plant growth and development (e.g. flowering and root growth) (Szabados and Savouré, 2010). Mattioli *et al.* (2009) suggested that proline may have a role in regulating cell division and embryogenesis in plants.

Increased light stress affects the production and accumulation of some metabolites in plants. For example, increasing light intensity is correlated with concentration of phenolics (Chalker-Scott and Fenchigami, 1989), taxol, baccatin III (Fett-Neto *et al.*, 1995) and anthocyanins (Chalker-Scott, 1999). Ramakrishna *et al.* (2011) reported that photoperiod regime influenced the level of indoleamine metabolites (serotonin and melatonin) in green algae. Long photoperiod treatments trigger the accumulation of ginsenoside metabolites in roots of *Panax quinquefolius*, a herbaceous perennial plant from the ivy family, compared to plants grown under short photoperiods (Li *et al.*, 1996). Accumulation of some metabolites has been reported to increase under salinity stress. For example, an increased level of polyphenols (Dixon and Paiva, 1995; Muthukumarasamy *et al.*, 2000), polyamines (Mutlu and Bozcuk, 2007) and total phenolics (Navarro *et al.*, 2006) was demonstrated in a variety of plants under salt stress. Accumulation of proline (Petrusa and Winicov, 1997) and anthocyanins (Parida and Das, 2005) in plants has been reported in salt tolerant species under salinity stress whereas decreased or regular levels of these metabolites were found in salt sensitive species (Petrusa and Winicov, 1997; Daneshmand *et al.*,

2010). Multifarious physiological roles such as photo-protection, antioxidative activity and contribution to osmotic adjustment are the main functions proposed for anthocyanins under adverse environmental conditions (Chalker-Scott, 1999; Gould, 2004). Increased concentrations of some metabolites such as flavonoids (Larson, 1988), anthocyanins (Chalker-Scott, 1999), saponins (Soliz-Guerrero *et al.*, 2002) and glycosides (Wang *et al.*, 2010a) have been reported in a variety of drought-stressed plants.

#### **1.1.4. Abiotic stress and organelles**

Abiotic stress affects the major subcellular organelles and most of these organelles contribute to primary defense responses and potentially become a source of ROS (Agrawal *et al.*, 2011). For example, chloroplasts, mitochondria, and peroxisomes are known to be the main sources of ROS since their normal metabolic function is reduction of oxygen or oxidation of water in cells (Moller *et al.*, 2007). An accumulation of ROS in organelles signals the stress to the nucleus to regulate the expression of genes encoding proteins such as proteases, antioxidant enzymes and heat shock proteins. The new proteins also contribute to the unfolding or refolding of damaged proteins or the degradation of impaired proteins (Taylor *et al.*, 2009).

Initially, the plasma membrane senses a stress using many receptor proteins embedded within it (Komatsu *et al.*, 2007). The plasma membrane is the most complex form of living membrane in a cell with a diverse protein structure that is determined by cell type, growth stage or environmental factors (Alexandersson *et al.*, 2004). It is known that under severe stress conditions, membrane permeability increases resulting in ion leakage from the membrane and impairment of membrane transport ability (Caldwell and Whitman, 1987). Environmental stresses can damage the physical structure of a membrane as well as its chemical properties. A deficiency in membrane integrity, for example as a result of peroxidation of membrane lipids under abiotic stress, enhances membrane permeability which can result in cell death. Moreover, abiotic stress can also negatively affect the stability and activity of membrane proteins (Huang, 2006).

It is generally assumed that the plasma membrane is the first site of environmental change perception and the second site are chloroplasts. The photosynthesis process is extremely sensitive to abiotic stress. Expectedly, chloroplasts, as the center of photosynthesis, are vital and important organelles in sensing abiotic stress signals initially perceived by the plasma membrane (Lee *et al.*, 2013). The redox state of a chloroplast changes during stress conditions and this influences the

expression of genes in the nucleus. For example, changes in redox state of plastoquinone, an electron carrier of PSII, regulates the expression of some nuclear and chloroplast encoded genes (mainly photosynthetic genes) under extreme conditions (Fernández and Strand, 2008). In chloroplasts, hydrogen peroxide and superoxide can form as a result of reduction of molecular oxygen at PSI and a strong reactive excited state of oxygen, singlet oxygen, can be produced in PSII. Generally, the main damage of stress to a chloroplast is linked to the ROS production in this organelle (Taylor *et al.*, 2009).

Various oxidases such as urate oxidase, xanthine oxidase, acyl-CoA oxidases and glycolate oxidase are located in peroxisomes. Peroxisomes use these enzymes to reduce oxygen to hydrogen peroxide while oxidizing other compounds (Reumann *et al.*, 2007). Peroxisomes are also involved in catabolism of xanthine which is a purine compound originating from DNA or RNA turnover (Corpas *et al.*, 1997). In addition to enzymatic antioxidants (e.g. SOD), the presence of some non-enzymatic antioxidants (e.g. ascorbate and glutathione) have been confirmed in leaf peroxisomes (Jiménez *et al.*, 1997). Accumulation of ROS in peroxisomes under stress conditions induces oxidative stress in the organelle which consequently affects the cell function (Taylor *et al.*, 2009). A number of reports suggest that under stress conditions, peroxisomes have different metabolic functions mediated by ROS such as inducing senescence (by leaf peroxisomes) or producing signal molecules in transduction pathways to increase the expression of defence genes (del Río *et al.*, 2003).

Proper folding of proteins is necessary for their function and transportation. Under environmental stress conditions, accumulation of unfolded and misfolded proteins in the endoplasmic reticulum (ER), is referred to ER stress (Deng *et al.*, 2013). In response to ER stress, a series of cellular mechanisms such as activation of unfolded protein response and ER-associated degradation are activated. These mechanisms contribute to alleviation of ER stress by inducing the production of factors that are involved in protein folding as well as removing the unfolded and misfolded proteins (Herczenik and Gebbink, 2008). In plants, the unfolded protein response of the ER has been found to be related to salinity, heat and drought stresses (Deng *et al.*, 2013). Mutation of factors involved in the ER stress response in plants resulted in hyper-sensitive phenotype under stress (Liu *et al.*, 2007) while the overexpression of some of these factors enhanced the plant stress tolerance (Fujita *et al.*, 2007).

## 1.2. Increased light intensity

Light is one of the essential factors of plant growth and development. However, it can be the source of ROS production and cell death (Wituszyńska and Karpiński, 2013). In plant cells, light energy is collected by pigments (mainly chlorophylls) and is transferred to reaction center complexes, PSI and PSII. Both photosystems are located in the thylakoid membrane of chloroplasts. Under increased light intensity, the rate of light absorption and transfer to the photochemical complexes will be affected. Since the photosystems are linked by a chain of electron carriers, any imbalance in light energy can result in uneven excitation of photosystems (Karpinski *et al.*, 1999). Excess light energy that cannot be used for carbon dioxide fixation and photosynthetic metabolism is called excess excitation energy (Gray *et al.*, 1996; Asada, 1999; Karpinski *et al.*, 1999). The term “excitation pressure of PSII” represents the relative reduction state of PSII. The energy imbalance resulting from an environmental stress such as increased light intensity leads to over-excitation of PSII (Gray *et al.*, 1996). Notably, excess energy is not only the consequence of high light stress, it can result from other abiotic stresses like high/low temperatures (Gray *et al.*, 1997) drought and salinity (Wituszyńska and Karpiński, 2013). An excess excitation energy enhances the electron transport rate and therefore the redox status of the photosynthetic electron transport chain changes. The redox change effects the regulation of chloroplast and nuclear genes such as those that encode light-harvesting complex proteins (Bailey *et al.*, 2001; Mishra *et al.*, 2012) and antioxidants (Karpinski *et al.*, 1997; Pfannschmidt *et al.*, 1999). Subsequently, a response process is triggered by redox signals from the photosynthetic electron transport chain, particularly the electron carrier, plastoquinone (Karpinski *et al.*, 1999; Mullineaux, 2000).

The phenomenon by which PSII is inactivated and damaged is called photo-inhibition (Barber, 1995; Ruban, 2009). Under increased light intensity when photo-inhibition occurs, excess excitation energy will excite PSII and the powerful oxidant PSII will oxidize the closest pigments and amino acids. The major target of this damage is the D1 protein which is a subunit of the multi-subunit PSII complex structure. Damaged D1 must be removed from the PSII complex and replaced by a new molecule (Barber *et al.*, 1994; Ruban, 2009; Gururani *et al.*, 2015). Light-dependent production of highly reactive oxygen derivatives is known as photooxidation (Foyer *et al.*, 1994). Although photooxidation is initially caused by absorption of excess light, it can also be induced by water deficit and salt stress (Reddy and Raghavendra, 2006). During photooxidation,

the excess amount of ROS can affect macromolecules such as lipids, nucleic acids and proteins and if a plant cannot prevent the photooxidative stress, eventually senescence and cell death will occur (Pinto-Marijuan and Munné-Bosch, 2014).

Several mechanisms are involved in protecting PSII from increased excitation pressure. Transition of energy from PSII to PSI or reduction of energy transfer to PSII by non-photochemical quenching are some examples of adjustment mechanisms (Huner *et al.*, 1998). However, over a long term of over-excitation of PSII, a plant might use mechanisms such as a reduction of antenna size, or induction of the capacity to use ATP and NADPH metabolically in order to maintain photochemical quenching of PSII (Huner *et al.*, 1998) and/or a change in leaf optical properties (e.g. through leaf epidermal or palisade cell properties to direct light) in order to decrease light absorption (Vogelman *et al.*, 1996).

An elevated excitation of electrons in antenna complexes (Chlorophyll-protein complexes in PSII and PSI) that absorbs the light, is inevitable under high light and the reaction centers of the photosystems will be saturated with energy (Asada, 1999; Karpinski *et al.*, 1999). To remove excess light energy, plants utilize the processes of photochemical and non-photochemical quenching. Photochemical quenching is defined as the processes of consumption of extra electrons through establishment of additional metabolic sinks. Photochemical quenching can be achieved by an enhancement of the photorespiratory rate, antioxidant activity of chloroplast enzymes and probably through increased metabolism of carbon and nitrogen. Another example is a reduction of oxygen in PSI by the Mehler reaction (Mullineaux and Karpinski, 2002). During the Mehler reaction, an oxygen molecule is reduced to  $O_2^-$  by an electron fed directly from PSI. This  $O_2^-$  is converted to  $H_2O_2$  by superoxide dismutase and then is converted to water by ascorbate peroxidase (Asada, 1999). Non-photochemical quenching affects the consumption of excess energy by transferring electrons to carotenoids where it is used as heat within the xanthophyll cycle. In this cycle, violaxanthin is converted to de-epoxidised zeaxanthin by violaxanthin de-epoxidase enzyme. This mechanism provides an important way to consume excess light energy in plants (Demmig-Adams and Adams, 2006).

Furthermore, in order to avoid high light stress, plants have also developed some morphological strategies such as alteration of leaf orientation, leaf folding, smaller leaf size and leaf thickness (Steyn *et al.*, 2002). Some internal strategies include plants' chloroplast movements, decreasing the number of photosynthetic reaction centers and increasing non-photosynthetic

pigments like anthocyanins (Mullineaux and Karpinski, 2002; Steyn *et al.*, 2002). Anthocyanins accumulation provides a protection for photosynthetic apparatus from excess light damage especially in leaves. It has been suggested that reduction of light intensity by anthocyanins can help plants to re-establish a light balance and to decrease the risk of photooxidative damage (Steyn *et al.*, 2002) since this pigment absorbs excess light photons which otherwise would be absorbed by the photosynthetic electron transport chain (Gould, 2004). Anthocyanins are the most common non-photosynthetic pigments that protect photosynthetic tissue under photoinhibition (Smillie and Hetherington, 1999).

### **1.3. Osmotic stress**

In order to maintain a positive turgor pressure, plant cells adjust their osmotic potential in balance with their water potential. Significant alteration in water potential in the environment, mainly by drought, salinity and cold stress, can cause osmotic stress in plants (Xiong and Zhu, 2002). Plants respond to osmotic stress with a wide range of mechanisms. The response might include morphological changes for example in root growth or developmental changes in the life cycle. Changes in uptake and transport of ions as well as metabolic adjustments (such as synthesis of osmoprotectants) are part of a plant response to an osmotic stress (Upadhyaya *et al.*, 2013). Compatible solutes play an important role in osmotic adjustment by decreasing the osmotic potential of cells to increase water absorption and maintain intercellular osmotic potential. Ion homeostasis is another important mechanism for plants to combat osmotic stress. Under osmotic stress, regulation of expression and/or activity of some ATPases, proteins of water channel (aquaporins) and ion transporters has been reported (Xiong and Zhu, 2002). Osmotic stress triggers the accumulation of ABA and ROS. Both ABA and ROS can be involved in osmotic stress cellular signaling networks in plants that in turn, activates various signaling proteins including transcription factors and protein kinases. These signaling proteins play signal transduction roles to help plants to respond to osmotic stress (Yoshida *et al.*, 2014).

### **1.4. Drought**

Among abiotic stresses, drought has been defined as one of the main environmental factors effecting plant growth, development and productivity (Boyer, 1982). Drought can damage plants physiologically, morphologically and biochemically (Parker, 1968). Physiological and



biochemical changes can result in a reduction in photosynthesis caused by limitation in CO<sub>2</sub> influx, carboxylation and activity of the electron transport chain (Akinci, 1997). Under drought, ROS scavenging compounds such as ascorbate and glutathione increase in cells. As well, ABA and compounds like proline, mannitol and sorbitol accumulate in drought stressed plants (Lichtenthaler *et al.*, 1981).

Drought and salinity stress induce similar responses since they both lead to dehydration of the cell and impairment of osmotic balance. Almost all plant physiology aspects and cellular metabolism alters under drought and salt stress (Liu and Zhu, 1998). The resulting signaling from these stresses controls three main parameters. First, in order to maintain cellular homeostasis, plants attempt to reinstate the cell osmotic and ionic balance. Second, in order to control the stress-induced damage, detoxification signaling is activated and third, cell division is regulated to inhibit plant growth (Zhu, 2002). Deceleration of cell division and inhibition of cell growth under drought stress is mainly regulated through the reduction of the activity of enzymes involved in cell division (Schuppler, 1998).

A plant response to drought stress is a sophisticated combination of strategies including: escaping from drought (e.g. accelerating life cycle); avoiding drought (e.g. deep rooting); drought tolerance (mainly by osmotic adjustment); resistance to drought (e.g. higher antioxidant content) and drought abandonment (e.g. losing old leaves) (Price *et al.*, 2002; Xu *et al.*, 2010; Debnath *et al.*, 2011). These strategies overlap with each other. For example, drought resistance can be achieved by a combination of drought avoidance, tolerance and escape mechanisms (Fang and Xiong, 2015).

Under mild and medium drought, plants can avoid the stress by morphological, growth and development changes. Stomatal closure, leaf rolling, accumulation of wax on the leaf in many plant species helps to reduce water loss (Fang and Xiong, 2015). In mature plants, root growth, density and root to shoot ratio might increase the ability of a root to take up water from deep soil layers (Mahajan and Tuteja, 2005). As drought stress progresses, these initial responses in plants are usually followed by drought escape mechanisms. Conversion from vegetative to reproductive phase in plants avoids complete failure to reproduce if the drought stress becomes severe (Fang and Xiong, 2015). Moreover, mature plants adjust their leaf area in response to the stress using precocious senescence and abscission of old leaves. (Mahajan and Tuteja, 2005). Drought escape can also be an artificial process of adjustment of planting, growth, life cycle to escape the local or

climatic drought (e.g. lack of rain, more moisture evaporation from lands) by farmers (Mitra, 2001). Drought tolerance is the ability of plants to maintain a level of physiological activities even under severe drought (Passioura, 1997). Enhancing osmoregulatory compounds to sustain cell turgor pressure is the main way to reduce stress damage (Fang and Xiong, 2015). Drought resistance refers to the combination of various mechanisms to change the resistance during developmental stages. It can be associated with antioxidant synthesis, photosynthesis and cell osmotic regulation at specific development stages. Due to the variability of a natural drought, plant species and developmental stage, evaluation of all drought resistance mechanisms can be difficult (Fang and Xiong, 2015).

One of the first responses of all mature plants to drought is reducing water lost through transpiration by closing stomata (Mansfield and Atkinson, 1990). Stomatal closure is called hydropassive closure when it is a direct result of water evaporation and not metabolically dependent. On the other hand, hydroactive closure occurs when stomatal closure is determined by metabolic processes and alteration of ion flux. Generally, metabolic dependent closure in plants is regulated by ABA. (Hartung *et al.*, 2002). Both modulation of ABA levels in the guard cells and long-distance transport of ABA can be involved in the regulation of stomatal closure (Wilkinson and Davies, 2002). Various processes can regulate ABA-induced stomatal closure, some of which are: synthesis of ABA versus catabolism in stressed roots; transferring ABA from roots into the xylem through apoplast or specialized xylem parenchyma cells; movement of newly-synthesized ABA with the transpiration in the leaf (around or inside of mesophyll cells) to the guard cells; converting inactive ABA (glycosylated ABA) to free ABA in the leaf apoplast (using  $\beta$  – glucosidase) and then to the guard cells (Davies *et al.*, 2002; Wilkinson and Davies, 2002). Moreover, as a chemical messenger, ABA can induce stomatal closure under drought stress mainly using secondary messengers such as protein kinases, ROS,  $\text{Ca}^{2+}$  and nitric oxide (Lim *et al.*, 2015). In addition, crosstalk between ABA and different hormones such as auxin, ethylene and cytokinins might be involved in root drought sensing and signaling in different plants (Janiak *et al.*, 2016).

Plants also respond to drought by a regulation of expression of many genes. Induced expression of hundreds of genes have been reported under drought. However, due to the complexity of a plant stress response, the specific functions of many of these genes have yet to be identified (Debnath *et al.*, 2011). It is known that some drought-inducible genes, encode proteins such as chaperones, mRNA-binding proteins, osmolyte biosynthesis enzymes, proline and sugar

transporters, proteins with regulation of signal transduction roles, proteases and enzymes for detoxification (Shinozaki and Yamaguchi-Shinozaki, 2007). Yamaguchi-Shinozaki *et al.* (1992) reported a number of independent cDNAs coding for drought-inducible proteins in *Arabidopsis*. These genes are called responsive to dehydration (RD) genes. Expression of three of these genes *RD29A*, *RD29B* (Yamaguchi-Shinozaki and Shinozaki, 1994.) and *RD26* (Fujita *et al.*, 2004) was also induced by exogenous ABA and other abiotic stress conditions such as salinity and low temperature. Therefore, expression analysis of these genes has been widely used as abiotic stress markers in plants (Neves-Borges *et al.*, 2012). It has been suggested that these stress marker genes function in the ABA response to abiotic stress or a change in cell osmotic potential (Yamaguchi-Shinozaki and Shinozaki, 1994; Fujita *et al.*, 2004).

### **1.5. Salinity stress**

High salinity is one of the most severe environmental stresses effecting croplands worldwide (Debnath *et al.*, 2011; Shavrukov, 2013). Salt stress is a term used for any kinds of salinity to which plants are exposed; however the major salt component affecting plants is NaCl (Shavrukov, 2013). Salinity can be caused by natural, or primary, reasons that result from accumulation of salts through natural processes over a long period of time. Secondary, or human-caused salinity is the result of unbalanced hydrology of the soil resulting from human activity (Parihar *et al.*, 2015).

Salinity stress induces osmotic and ionic stresses in plants. Osmotic stress is the first component of salt stress that occurs immediately after contact of the root with unfavourable levels of salt in the soil (Shavrukov, 2013). The osmotic potential of salt in the soil decreases the ability of the plant to take up water. Osmotic stress is also the first stage of drought stress which similarly leads to accumulation of osmolytes and a change in stomatal conductance (James *et al.*, 2008). Therefore, in cases of cellular and metabolic changes, drought and salinity have many responses in common (Parihar *et al.*, 2015). When cells characterize the relative differences in their osmotic pressure with the outside environment, osmotic stress occurs. Plants under osmotic stress adjust the osmotic potential inside and outside of their cells. There are different mechanisms for plants to sense the change in turgor pressure. Some examples of these mechanisms are: activation of kinase receptors, accumulation of compounds involved in deactivation of nonselective cation

channels and increasing plasma membrane proteins responsible for enhancing  $\text{Ca}^{2+}$  influx (Julkowska and Testerink, 2015).

The second component of salt stress is ionic stress which follows the osmotic response with a time delay. The delay is because of the time that is needed for  $\text{Na}^+$  ions to reach a toxic concentration in the cytoplasm (Shavrukov, 2013). An ionic stress follows an osmotic stress when the level of cytoplasmic  $\text{Na}^+$  ions rises to toxic levels (Shavrukov, 2013). It is suggested that ionic stress is signaled through the salt overly sensitive (SOS) signaling pathway. Under an excess amount of  $\text{Na}^+$ , the SOS pathway triggers a  $\text{Ca}^{2+}$  signal in the cytoplasm that results in a broad change in expression and activity of ion transporters (Zhu, 2002). The SOS pathway has three components in Arabidopsis. SOS1 controls the transport of  $\text{Na}^+$  from roots to shoots, SOS2 is a protein kinase and probably is part of signaling network between the salt stress response and ROS, and SOS3 acts as a calcium-binding protein, with a role as a sensor of salt stress and excess cytoplasmic ions. (Ji *et al.*, 2013).

Seed germination is a critical stage of a plant's life cycle and an important stage for ensuring seedling establishment and early plant growth. When seeds are planted under severe saline conditions, they cannot imbibe water due to the lower osmotic potential of the media containing salt. This will affect protein metabolism, hormonal balance and disturb the metabolic and physiological processes of the plant (Dantas *et al.*, 2007; Parihar *et al.*, 2015). During growth, salinity stress has a highly impact on plant growth rate. The effect of salinity on plants can occur through a two-phase process. First,  $\text{Na}^+$  and  $\text{Cl}^-$  ions increase in plant cells and osmotic stress occurs. Consequently, shoot growth and leaf expansion is reduced and the formation of lateral buds is inhibited. Secondly, harmful amounts of these ions, mainly  $\text{Na}^+$ , accumulate in plant leaves and inhibit photosynthesis.  $\text{Na}^+$  is the principal ion causing physiological harm, it impairs stomatal regulation by disturbing the uptake of  $\text{K}^+$  while it also disturbs the turgor pressure, pH and the activity of cytoplasmic enzymes (Tavakkoli *et al.*, 2011; Parihar *et al.*, 2015).

ABA increases in plants in response to salinity stress. The increase in ABA level activates ABA-dependent protein kinases and these proteins target transcription factors. In turn, the transcription factors regulate the expression of genes involved in cellular detoxification pathways, acclimation responses and osmotic protection mechanisms (Deinlein *et al.*, 2014). In response to salt stress, organic osmolytes such as proline, polyamines and glycine betaine accumulate in cells to maintain the intercellular osmotic potential and prevent salt damage (Verslues *et al.*, 2006).

Furthermore, some of these osmolytes have been proposed to contribute in recovery from salinity stress. For example, during recovery, proline plays a role as a signalling molecule in the regulation of cell proliferation, cell death and stress-recovery gene expression (Szabados and Savoured, 2010).

## **1.6. Purine-derived ureide metabolism**

In 1838, Liebig and Wohler showed that ureides could be considered as the derivatives of the acylation of urea (Pope, 1919). Between 1882 and 1906, Emil Fischer showed that adenine and xanthine (from vegetables), uric acid and guanine (from animal excretion) and caffeine, all belong to one homogeneous family named purines and they could be derived from each other. Since then, various hydroxyl and amino derivatives of the heterocyclic aromatic compound, purine, have been identified (Rosemeyer, 2004; Legraverend and Grierson, 2006). Purines are known to be the most widely distributed nitrogenous heterocyclic structure in nature (Rosemeyer, 2004).

In most primates, including humans, uric acid is the major end product of purine metabolism because they lack uricase to further oxidize urate (Serventi *et al.*, 2010). In fact, uric acid is excreted as a waste material because mammalian cells are only capable of partial degradation of purines. In contrast, nitrogen is a main limiting nutrient for plant growth and plants degrade purines completely to recapture the nitrogen, using it to synthesize other nitrogenous compounds (Montalbini, 1992; Stasolla *et al.*, 2003; Werner and Witte, 2011). In plants, recycling purines through metabolic remobilization of nitrogen from source organs (ex: old leaves) to sink organs (ex: seeds) recycles nitrogen within the plant (Werner and Witte, 2011).

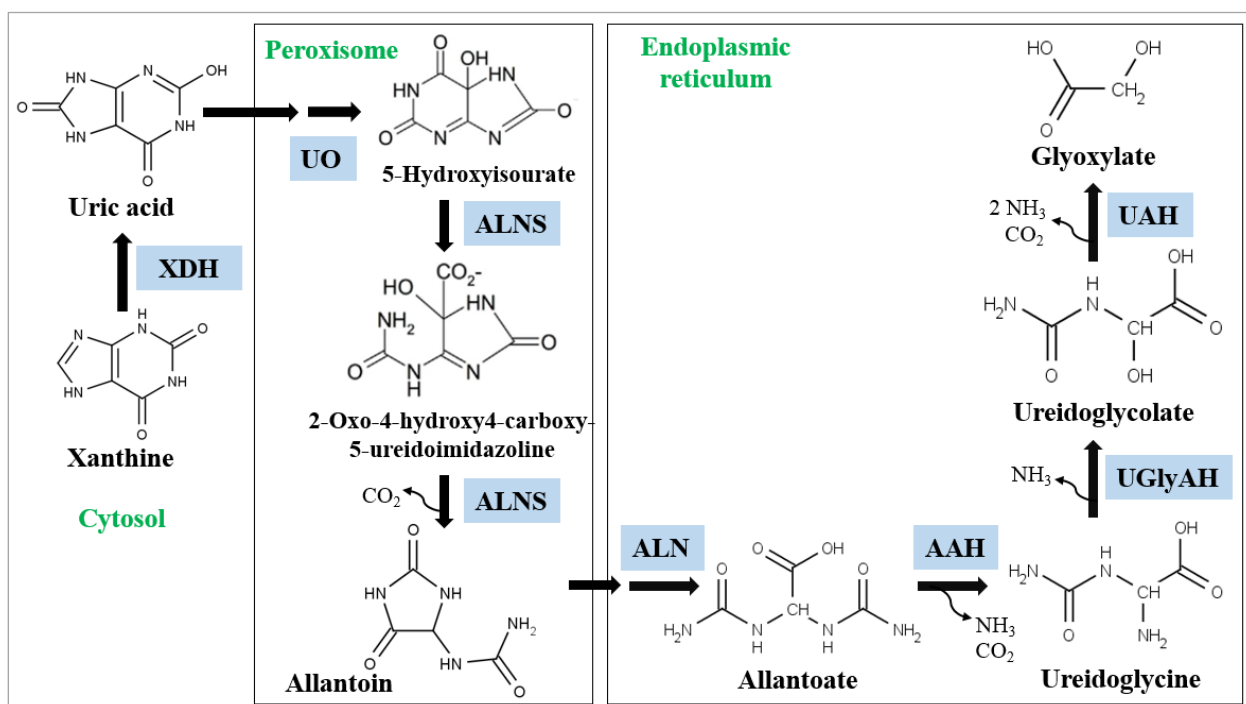
Ureide metabolism in *Arabidopsis* begins with the generation of xanthosine from guanosine by a guanosine deaminase (E.C. 3.5.4.15), followed by hydrolysis of xanthosine to xanthine via a nucleoside hydrolase (Dahncke and Witte, 2013). Xanthine is the first compound in ureide metabolism and subsequently it is oxidized to uric acid by xanthine dehydrogenase (XDH; E.C. 1.17.1.4) (Figure 1.1) (Triplett *et al.*, 1982). In *Arabidopsis*, two xanthine dehydrogenase genes (*XDH1* and *XDH2*) have been identified and cloned (Hesberg *et al.*, 2004). Uricase (urate oxidase, UO; E.C. 1.7.3.3) converts uric acid to 5-hydroxyisourate (5-HIU) (Hanks *et al.*, 1981; Ramazzina *et al.*, 2006). In the reaction that 5-HIU is metabolized to allantoin, a 2-oxo-4-hydroxy-4-carboxy-5-ureido-imidazoline (OHCU) intermediate and one molecule of CO<sub>2</sub> is released. In *Arabidopsis*, this is accomplished by a single bifunctional enzyme, allantoin synthase (ALNS; E.C.

4.1.1.97; 3.5.2.17) (Kim *et al.*, 2007; Lamberto *et al.*, 2010; Pessoa *et al.*, 2010). The breakdown of allantoin to allantoate, is catalyzed by allantoinase (ALN; E.C. 3.5.2.5) (Yang and Han, 2004) and allantoate is metabolized to ureidoglycine by allantoate amidohydrolase (AAH; E.C. 3.5.3.4) and resulting one CO<sub>2</sub> and one molecule of ammonia (Todd and Polacco, 2006; Werner *et al.*, 2008). Ureidoglycine is degraded to ureidoglycolate and a molecule of ammonia by ureidoglycine aminohydrolase (UGlyAH; E.C. 3.5.3.26) (Serventi *et al.*, 2010) and in the final step, ureidoglycolate is finally hydrolyzed to glyoxylate, CO<sub>2</sub> and two molecules of ammonia by ureidoglycolate amidohydrolase (UAH; E.C. 3.5.1.116) as well as a non-enzymatic reaction (Werner *et al.*, 2010). Figure 1.1 shows the metabolism of ureides in plants. Oxidation of xanthine to uric acid takes place in the cytosol. The enzymatic steps that convert uric acid to allantoin occur in the peroxisome. The rest of the reactions to the end product, glyoxylate, occurs in the endoplasmic reticulum (Werner and Witte, 2011).

### 1.6.1. Ureide transporters

Ureides are important compounds in nitrogen transportation in plants. Identification and functional analysis of their transporters is important as ureides are likely to play roles in plant growth, reproduction, metabolic regulation and nitrogen transportation. However, currently only a few ureide transporters have been studied in plants (Tegeder, 2014).

Initially, a transporter of adenine and guanine, named AtPUP1, was assumed to transport purine derivatives in Arabidopsis (Gillissen *et al.*, 2000). Argyrou *et al.*, (2001) reported that the Lpe1 (leaf permease1) transporter with a function in chloroplast development is involved in transportation of uric acid and xanthine in maize. Desimone *et al.* (2002) reported the first transporter of allantoin in plants as AtUPS1 in Arabidopsis. UPS1 is from a protein superfamily all of which have a number of predicted membrane-crossing domains. This family consists of five conserved members that are also present in other plant species including rice, tomato and cowpea (Desimone *et al.*, 2002). Xanthine, uric acid as well as the oxo-derivation of the purine or imidazol rings (nitrogen compounds with similar heterocyclic feature) were suggested as putative AtUPS1 substrates (Desimone *et al.*, 2002). Following this report, PvUPS1 was found to be the allantoin transporter in nodules of a legume plant, common bean (*Phaseolus vulgaris*) (Pelissier *et al.*,



**Figure 1.1. The main steps of ureide biosynthesis and catabolism in *Arabidopsis* in cytosol, peroxisome and endoplasmic reticulum.** Arrows show the direction to the conversion of each compound to the next compound by ureide metabolic enzymes. Four  $\text{NH}_3$  molecules and three molecules of  $\text{CO}_2$  are released in the pathway. The enzymes are shown in blue boxes; as: XDH, Xanthine Dehydrogenase; UO, Uricase; ALNS, Allantoin Synthase; ALN, Allantoinase; AAH, Allantoate Amidohydrolase; UGlyAH, Ureidoglycine Aminohydrolase; UAH, Ureidoglycolate Amidohydrolase;  $\text{NH}_3$ , Ammonia

2004). PvUPS1 is able to transport allantoin, uric acid and xanthine but not the downstream products of allantoin including allantoate and urea. However, allantoin seems to be the predominant substrate for PvUPS1 and the other ureide metabolites probably are not main substrates of UPS1 in beans. It remains to be determined if xanthine and uric acid are transported by PvUPS1 and whether their transportation varies with physiological conditions (Pelissier *et al.*, 2004). Regarding the transport mechanism of AtUPS1, available data suggest a transportation without ATPase activity. However, since both AtUPS1 and PvUPS1 have a conserved motif for ATP binding, further studies are needed to address the function of this motif (Desimone *et al.*, 2002; Pelissier *et al.*, 2004).

In 2012, Collier and Tegeder reported that GmUPS1-1 and GmUPS1-2 are transporters of both allantoin and allantoate in roots of soybean (*Glycine max* L.). The genes coding these two similar proteins might be a product of gene duplication in soybean (Schmutz *et al.*, 2010). The GmUPS1-1 and GmUPS1-2 are localized in the plasma membrane of soybean cells (Collier and Tegeder, 2012).

On a cellular level, it is still not known if uric acid enters peroxisomes through selective channels or via specific transporters. It is also unknown how allantoin is translocated to the endoplasmic reticulum. There are several possibilities including transport through the cytosol using transporters or maybe through peroxisome to endoplasmic reticulum vesicles (Werner and Witte, 2011).

## **1.7. Historical Overview of ureides in plants**

### **1.7.1. 1930s-1970s**

The earliest documentation of ureides allantoin and allantoate as nitrogenous compounds in plants was in 1938 by Brunel and Echevin (cited by Thomas and Schrader, 1981). That ureides are generated from purine degradation in plants has been well documented (Tracey, 1955). Between 1952 and 1956, a group of German scientists studied the physiological role of ureides, allantoin and allantoate in a number of plant species. First, they found high amounts of ureides, allantoin and allantoate in roots and stems of some plants like the herbal medicine, Comfrey (*Symphytum officinale* L.) from the *Boraginaceae* family, a few trees and shrubs from a small family of *Hippocastanaceae*, maple from *Aceraceae* (or Maple family) and various number of



plants from *Leguminous* (or Fabaceae family) (Mothes and Engelbrecht, 1952; as cited by Mothes, 1961). It was suggested that in these plants, ureides are mainly found in xylem and they transform to proteins and amino acids in the growing tips during spring and then remobilize from leaves to the storage organs in autumn (Mothes and Engelbrecht, 1952, 1954; as cited by Mothes, 1961). Interestingly, higher levels of ureides were observed in response to dark treatment of detached *Phaseolus* leaves (Mothes, 1956; as cited by Mothes, 1961). In addition, when these darkened leaves were kept at high temperature (e.g., 32°C), the ratio of ureides to amides increased significantly compared to 4°C. Therefore, the authors suggested that ureide and amide formation and their ratio follows a specific carbohydrate economy (Mothes, 1956; as cited by Mothes, 1961). In another experiment, detached *Phaseolus* leaves treated with auxin resulted in formation of a root system. Large amount of allantoin was generated from uptake of nitrogen by roots which then was delivered to the growing leaves (as source of nitrogen). The same pattern was observed in cut off leaves of comfrey (*Symphytum* genus from *Boraginaceae* family) where ureides were accumulated more than 100 times in response to nitrogen shortage in the growing detached tissue (Mothes and Engelbrecht, 1956; as cited by Mothes, 1961). Bollard (1956) reported the presence of ureides in the xylem sap of plant species from 23 families. Low levels of ureides were found in some of these species but in others (ex. *Acer* and *Persea* genera) a high levels of ureides, mainly allantoate was observed. Mothes (1961) suggested that ureides might have a main role in translocation of nitrogen from roots in plants. In 1962, all the available information about ureides were summarized by Reinbothe and Mothes, underlining the importance of allantoin and allantoate's role in the metabolism of some plants by translocation and storage of nitrogen.

### **1.7.2. 1970s- 1995: Ureides in tropical legumes**

Although ureides had already been identified in higher plants, the accumulation of allantoin in legumes was first reported in soybean roots and nodules by Ishizuka *et al.* (1970). However, non-nodulated soybean plants only had low levels of allantoin (Ishizuka *et al.*, 1970; Matsumoto *et al.*, 1975). In N<sub>2</sub>-fixing legumes, organic nitrogen generated from symbiotic N<sub>2</sub> fixation by soil bacteria and root nodules can be transported to the xylem either as amides or ureides. Ureides were found to be the dominant transporting compounds in many tropical legumes (Atkins *et al.*, 1982). Various research showed the accumulation of ureides in different legumes and indicated this nitrogen metabolism in a group of tropical legumes including *Glycine*, *Vigna* and *Phaseolus* spp.

(Rawsthorne *et al.*, 1980; Shelp *et al.*, 1983., Peoples *et al.*, 1985; Schubert, 1986; Corby, 1988; Kouchi and Higuchi, 1988; Day and Copeland, 1991; Streeter, 1992). Christensen and Jochimsen (1983) reported higher activity of ureide synthesis enzymes in soybean compared to pea as expected considering the very high levels of ureides in xylem sap of the ureide transporter legume, soybean. Later, ureide metabolism was specifically studied in soybean leaves (Shelp and Ireland, 1985) and shoots (Shelp and Da Silva, 1990) to indicate the path of ureide assimilation and organ specific distribution/translocation of ureides, respectively. In 1997, a taxonomic and evolution study of all legumes using the phylogeny of a chloroplast-encoded gene (ribulose-1-5-bisphosphate carboxylase/oxygenase, *rbcL*) showed that all legumes with high levels of ureides belong to a group of legumes called tropical legumes. In all other legumes, translocation of the fixed nitrogen was limited to amides (Doyle *et al.*, 1997).

#### **1.7.3. 1995-2005: Ureides in tropical legumes under water stress**

In the second half of the 1990s, accumulation of allantoin and allantoate under drought and water-limitation stress was reported in nodules and shoots of a number of ureide transporting legumes (Sinclair and Serraj, 1995; Serraj and Sinclair, 1996; Serraj *et al.*, 1999). The reports suggested that high levels of ureide was the result of nitrogen fixation inhibition under drought. These observations were in parallel with a previous report on the effect of allopurinol, an inhibitor of XDH, on reducing the metabolism of ureides in soybean (Fujihara and Yamaguchi, 1978). Vadez *et al.* (2000) reported that asparagine or/and ureides might be involved in the inhibition of nitrogen fixation in these legumes. This hypothesis was supported by Purcell *et al.* (2000) when drought tolerant soybean plants accumulated less ureides in leaves than drought sensitive plants under both well-watered and drought conditions. In general, since drought stress prevents nitrogen-fixation in soybean, it was proposed that ureide accumulation is the feedback of nitrogen fixation inhibition in nodules under unfavorable environment conditions (Vadez *et al.*, 2000; Serraj *et al.*, 2001).

#### **1.7.4. After 2005: Ureides and nitrogen fixation under abiotic stress**

The link between ureide accumulation and suppression of nitrogen fixation under stress was questioned when increased concentrations of ureides are observed in both nodulated and non-nodulated soybean subjected to drought stress (King and Purcell, 2005). Following this,

accumulation of ureides was reported in nodules of both tolerant and sensitive cultivars of soybean at the late stage of drought (Ladrera *et al.*, 2007). However, during early drought stress, no accumulation of ureides was detected in the leaves. The authors concluded that the levels of ureide in leaves is not relevant to nitrogen fixation inhibition under drought. But they might have a role in the late stage of a severe drought which has still to be explained (Ladrera *et al.*, 2007). Alamillo *et al.* (2010) observed that ureide concentration was enhanced in common bean on day 7 and then more so on day 14 after drought treatment, whereas for example nitrogen fixation was inhibited 50% on day 7 of withholding water. This observation and the increased number of senesced nodules after withholding water led researchers to consider the possibility of ureide accumulation even in the absence of functional nodules. Therefore, they tested the effect of water stress on ureide level of nodulated and non-nodulated common bean plants and found an increase in ureide content in root, leave and shoot tissues in both types of plants (Alamillo *et al.*, 2010). Coletto *et al.* (2014) reported the accumulation of ureides in shoots and leaves of common bean (*Phaseolus vulgaris*) and not in the nodules under prolonged drought condition. In their study, higher concentrations of ureides in aerial parts of the plants were found days after the senescence of nodules in very sensitive genotypes under drought stress (Coletto *et al.*, 2014). Taken together, it was suggested that ureide accumulation is uncoupled from nitrogen fixation and could be a general stress response in legumes (Alamillo *et al.*, 2010; Coletto *et al.*, 2014). Since 2005 and especially during the last couple of years, accumulation of ureides has been observed in a number of non-legume plants under various abiotic stress conditions, questioning the relevance of ureide accumulation to nitrogen fixation under stress.

#### **1.7.5. Ureides in non-legume plants under abiotic stress**

Accumulation of allantoin has long been recognized in the well-known medicinal plant, comfrey (*Symphytum officinale* L.), and this compound has been suggested to contribute to the pharmacological effects and medical efficacy of comfrey (Sousa *et al.*, 1991; cited by Mazzafera *et al.*, 2008). Castro *et al.* (2000) reported that the accumulation of allantoin increased in comfrey roots in response to long photoperiod. Increasing the photoperiod from 8 to 12, 16 and 20 hours per day, resulted in an increase in the concentration of allantoin in roots 60 days after the treatments. Ureides were reported to accumulate under prolonged dark treatment in *Arabidopsis* (Brychkova *et al.*, 2008). Dark treatment did not modify the level of xanthine but it increased the

concentration of allantoin and allantoin significantly. Dark treatment increased the transcript levels from genes coding for ureide synthesis (e.g. *XDHI* and *UO*) and decreased the transcription of genes encoding enzymes that catabolize ureides (e.g. *ALN* and *AAH*) (Brychkova *et al.*, 2008). Malik *et al.* (2016) reported that *Eutrema salsugineum* plants grown under high light had 2.5-fold more total ureides (allantoin, allantoate, ureidoglycolate and glyoxylate) compared to plants grown under moderate light.

There has been some indication that allantoin is involved in dehydration stress or drought response in plants. Higher levels of allantoin were observed in *Arabidopsis* seedlings exposed to dehydration shock (Watanabe *et al.*, 2014a). In rice, the concentration of allantoin was found to be positively correlated with the drought tolerance of the genotypes (Degenkolbe *et al.*, 2013). Oliver *et al.*, (2011) investigated the stress metabolic response of desiccation tolerant genotypes of *Sporobolus* (a species from grass family) compared to the desiccation sensitive genotype. Under full hydration conditions, the desiccation tolerant genotype (*Sporobolus stapfianus*) had eight-fold more allantoin than the sensitive one (*Sporobolus pyramidalis*). Under dehydration stress, allantoin accumulated to high levels in the desiccation tolerant plants mainly during late stages of stress (Oliver *et al.*, 2011). Yobi *et al.* (2013) suggested that allantoin has a role in desiccation survival and recovery in *Selaginella lepidophylla* (a desiccation tolerance species of spike moss which has the ability to survive a full desiccation state) since it was significantly more abundant in plants under partial or fully dehydrated conditions (Yobi *et al.*, 2013).

Recently, a number of studies reported that salt stress increases the ureide level in plants. Nam *et al.* (2015) observed a high accumulation of allantoin in rice roots in response to long-term mild salt stress. High accumulation of allantoin was also observed in rice seedling roots in response to salt and salt/ABA treatments (Wang *et al.*, 2016). Significant amounts of allantoin and allantoate were also observed in the halophyte plant, *Salicornia europaea* L. (Ventura *et al.*, 2010). Following this observation, Ventura *et al.* (2014) further detected higher accumulations of allantoin and allantoate in a few genotypes of the halophyte plant, *Crithmum maritimum*, in response to irrigation with 50 and 100 mM NaCl.

The level of allantoin in rice grains of different genotypes showed a positive correlation with seedling survival under low temperature and drought stress (Wang *et al.*, 2012). A positive correlation between levels of allantoin and hydrogen peroxide as well as the level of root damage in rice exposed to zinc deficiency or excess bicarbonate has been reported (Rose *et al.*, 2012).

Table 1.1 summarizes the main reports in non-legume plant species that contained higher levels of ureides under a variety of abiotic stresses.

### **1.8. Putative functions of ureides during plant stress**

The end product of purine metabolism in humans, uric acid, is an endogenous antioxidant. It is well known that uric acid has beneficial effects in human cells because of scavenging harmful radicals (Grune *et al.*, 2005). Due to the loss of activity of uricase which is the enzyme that converts uric acid to allantoin, uric acid is the end product of purine metabolism in humans. Lack of ability to metabolize uric acid has been considered to be a result of evolutionary selection of uricase mutants in human which probably represents the antioxidant function of uric acid (Wu *et al.*, 1992). As a hypothesis, similar to the other purines, uric acid is a strong electron donor and its concentration in hominids may enhance the defense against oxidative stress conditions (Proctor, 1970). Uric acid has been found to be one of the most capable water-soluble antioxidants in human plasma (Yeum *et al.*, 2004). It has been reported that singlet oxygen ( $^1\text{O}_2$ ) can be scavenged more effectively by urate than other antioxidants, such as ascorbate, in plasma (Ames *et al.*, 1981). In humans, allantoin is formed by the non-enzymatic oxidation of uric acid probably in response to high level of some ROS like hydrogen peroxide. When human disease is associated with elevated oxidative stress, the higher oxidative turnover of uric acid results in an increased amount of allantoin in plasma (Kandar, 2016). During the last decade, clinical studies on human disease have proposed that the non-enzymatic formation of allantoin may be a sensitive biomarker of oxidative stress *in vivo*. Therefore, allantoin has been suggested to be a useful indicator of oxidative stress with potential clinical applications (Yardim-Akaydin *et al.*, 2006; Kandar and Kova, 2008; Il'yasova *et al.*, 2010). Gus'kov *et al.* (2001) investigated the effect of allantoin on SOD and CAT antioxidant enzymes using nonpurebred white male rats that were injected with allantoin before exposure to an elevated pressure of oxygen (hyperbaric oxygenation) to induce oxidative stress. Pretreatment with allantoin positively affected the activity of antioxidants indicating that allantoin may have the ability to modulate or induce the activity of these antioxidant enzymes under oxidative stress situations (Gus'kov *et al.*, 2001).

**Table 1.1. High ureide concentration in non-legume plants under various abiotic stresses presented in order of discovery.**

<b>Plant species</b>	<b>Abiotic stress</b>	<b>Ureide compound(s)</b>	<b>Reference</b>
<i>Arabidopsis thaliana</i>	Prolonged darkness	Allantoin, Allantoate	Brychkova <i>et al.</i> , (2008)
<i>Sporobolus stapfianus</i> and <i>Sporobolus pyramidalis</i>	Desiccation	Allantoin	Oliver <i>et al.</i> , (2011)
Rice ( <i>Oryza sativa</i> )	Zinc deficiency and excess bicarbonate	Allantoin	Rose <i>et al.</i> , (2012)
Coffee ( <i>Coffea arabica</i> )	High light	Allantoin	Pompelli <i>et al.</i> , (2013)
<i>Selaginella lepidophylla</i>	Desiccation	Allantoin	Yobi <i>et al.</i> , (2013)
Rice ( <i>Oryza sativa</i> )	Drought	Allantoin	Degenkolbe <i>et al.</i> , (2013)
<i>Arabidopsis thaliana</i>	Dehydration shock	Allantoin	Watanabe <i>et al.</i> , (2014a)
<i>Crithmum maritimum</i>	Salinity	Allantoin, Allantoate	Ventura <i>et al.</i> , (2014)
Rice ( <i>Oryza sativa</i> )	Salinity	Allantoin	Nam <i>et al.</i> , (2015)
Rice ( <i>Oryza sativa</i> )	Salinity	Allantoin	Wang <i>et al.</i> , (2015)
<i>Eutrema salsugineum</i>	High light	Allantoin, Allantoate, Ureidoglycolate, Glyoxylate	Malik <i>et al.</i> , (2016)
<i>Arabidopsis thaliana</i>	Salinity	Allantoin	Lescano <i>et al.</i> , (2016)

Regarding plants, the possibility of involvement of ureides in ROS scavenging was first reported by Brychkova *et al.* (2008). Ureides, allantoin and allantoate were suggested to act as cell ROS protectants in response to prolonged dark treatment since their exogenous application decreased ROS accumulation in Arabidopsis leaves (Brychkova *et al.*, 2008). Brychkova *et al.* (2008) also showed that mutation of the first enzyme of the ureide metabolism pathway, XDH1, resulted in increased premature senescence, higher levels of ROS and enhanced chlorophyll degradation in leaves. Watanabe *et al.* (2010) reported that the simultaneous silencing of *XDH1* and *XDH2* in RNAi (RNA interference) transgenic lines of Arabidopsis, negatively influenced seedling biomass as well as cell survival rate under drought stress. Transgenic plants also accumulated higher levels of H<sub>2</sub>O<sub>2</sub> than wild-type, suggesting that the intermediates of ureide metabolism might provide an antioxidative mechanism (Watanabe *et al.*, 2010). The same transgenic lines exhibited significantly more sensitivity to oxidative stress compared to wild-type when germinated under superoxide-generating paraquat containing media (Watanabe *et al.*, 2014b). Higher concentrations of a number of compounds including allantoin were found in rice roots under a combination of both zinc deficiency and high bicarbonate concentration. The increase in concentration of allantoin showed a positive relationship with an increase in hydrogen peroxide level as well as root damage, indicating a likely role for allantoin in ROS production and/or response (Rose *et al.*, 2012). Wang *et al.* (2012) reported that allantoin did not serve as an antioxidant and did not have significant effect on scavenging free radicals *in vitro*.

On the other hand, Watanabe *et al.* (2014a) reported that increased levels of allantoin elevates ABA levels, and as a result, increases stress tolerance in Arabidopsis. In fact, a possible regulatory role was proposed for allantoin to effect ABA production in Arabidopsis in response to stress conditions (Watanabe *et al.*, 2014a). Later, this group also reported that purine degradation metabolites may have stress response roles by other mechanisms. Exogenous application of both uric acid and allantoin increased the mRNA levels of the proline biosynthesis enzyme, *P5CS*, in wild-type Arabidopsis in the absence of abiotic stress (Watanabe *et al.*, 2014b). Since proline is a cellular protectant under stress, it was suggested that uric acid and allantoin or their metabolites might contribute to plant acclimation to environmental changes (Watanabe *et al.*, 2014b).

## 1.9. Research questions

The overall aim of my Ph.D. research was to study ureide metabolism in response to abiotic stress in *Arabidopsis thaliana*. The objective of my thesis is to address the following research questions and test the associated hypotheses:

1. **Ureide accumulation under abiotic stress.** Ureide (allantoin and allantoate) accumulation in nitrogen-fixing legumes under drought stress had been reported abundantly. Several reports suggested that increased levels of ureides in these legumes is associated with nitrogen fixation inhibition in the nodules under drought (Serraj *et al.*, 1999; Vadez *et al.*, 2000; Serraj *et al.*, 2001). However, a number of reports and results presented by Alamillo *et al.* (2010), questioned the involvement of ureides in nitrogen fixation inhibition. Alamillo *et al.* (2010) suggested that accumulation of ureides in common bean under drought stress is a general plant response to stress that is probably regulated at the transcriptional level. Higher levels of ureides were also found in wild-type *Arabidopsis* in response to dark stress and senescence treatments (Brychkova *et al.*, 2008). These reports lead to the question: does ureide level increase in *Arabidopsis* under abiotic stress conditions? I hypothesize that environmental stresses will increase the level of some ureide compounds and as a result the expression of related genes in *Arabidopsis*. Therefore, I investigated the changes in concentration of ureides and expression of ureide genes in wild-type *Arabidopsis* in response to abiotic stresses. The abiotic stresses that were used for this purpose were drought, increased light intensity in mature plants and NaCl/mannitol treatments in seedlings.

2. **The role of ureide metabolic enzymes during abiotic stress.** Increased levels of ROS have been detected in *XDH*-suppressed *Arabidopsis* mutants after exposure to drought. However, when plants were subjected to the medium supplemented with uric acid, the mutant phenotype was indistinguishable from that of wild-type plants (Watanabe *et al.*, 2010). Therefore, it was suggested that the metabolites of ureide metabolism play a role in drought acclimatization of *Arabidopsis* (Watanabe *et al.*, 2010). This leads to the question: are mutants in various ureide metabolic genes more sensitive or tolerant to abiotic stress? I hypothesize that mutation (T-DNA insertion mutants) in ureide metabolic enzymes results in different phenotypes under abiotic stress when compared to wild-type plants. My prediction is that mutants in ureide biosynthesis (*xdh* and *uo*) will show a



sensitive phenotype due to lack of allantoin and allantoate accumulation. But the mutants in ureide catabolism (*aln* and *aah*) will show a tolerant phenotype due to ureide accumulation. Therefore I screened and isolated a number of homozygous T-DNA knock-out lines deficient in ureide metabolic enzymes and assessed their response to the abiotic stress conditions listed above.

**3. Stress response of *aln* mutants with high levels of allantoin.** When Arabidopsis mutants of *XDHI* were treated under dark stress, they showed higher levels of ROS and increased mortality rate in comparison to wild-type plants. However, the amount of ROS and mortality rate were decreased in the mutants in the presence of exogenous allantoin and allantoate (Brychkova *et al.*, 2008). This report and the preliminary observations of the previous hypothesis of the present research, lead to the question: does allantoin increase abiotic stress tolerance in Arabidopsis? I hypothesize that T-DNA insertion mutant, *Ataln*, that accumulate high levels of allantoin exhibit higher abiotic stress tolerance and less accumulation of ROS in comparison to wild-type. I tested the tolerance of *Ataln* seedlings to NaCl and adult plants to drought and increased light stresses compared to the wild-type using a number of physiological measurements. I also measured  $O_2^-$  and  $H_2O_2$  levels in mutant and wild-type leaves under drought stress. Further, I hypothesized that an exogenous application of allantoin will increase wild-type seedling tolerance to NaCl stress. This hypothesis was tested by exogenous application of two concentrations of allantoin to 100 mM NaCl plates.

Overall, this dissertation contains five sections in the result section, Chapter 3. In the first section of the results (3.1), I have explained the characterization of homozygous T-DNA insertion mutants of the ureide metabolic enzymes and phenotypic comparison of the mutants with wild-type plants under some abiotic stress conditions such as increased light. In parts 3.2 and 3.3, I have covered the results of drought and increased light intensity experiments. In section 3.4, the ureide concentration and gene expression in wild-type seedlings exposed to NaCl and mannitol treatments is shown. In the final two result parts, I have described the response of *Ataln* seedlings to NaCl stress (3.5) as well as the effect of exogenous application of allantoin on tolerance of wild-type Arabidopsis seedlings to NaCl stress (3.6).

## CHAPTER 2. MATERIALS AND METHODS

### 2.1. Plant material and growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used as wild-type plant material in all of the experiments in this research. Arabidopsis seed sterilization and growth conditions were based on procedures suggested by the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA; <https://abrc.osu.edu>). Seeds were sterilized for 8 min in commercial bleach (Sodium hypochlorite) containing 0.05% (w/v) Tween-20 and then rinsed 3 times with sterile water. In all of the experiments, half-strength Murashige-Skoog (MS) was used to germinate seeds. The media contained Murashige-Skoog basal salts (PhytoTechnology Laboratories, KS, USA), 0.8% (w/v) agar (Sigma, St. Louis, MO, USA) and 1% (w/v) sucrose (BioShop, Burlington, ON, Canada) (Murashige and Skoog, 1962). The pH of MS media was adjusted to 5.7. To adjust the pH of media or solutions to the desired pH, a pH-meter (SevenEasy, Mettler Toledo, USA) and 1 M NaOH or 1 M HCl was used.

Disposable sterile round (100 x 15 mm) or square (100 x 100 x 15 mm) petri dishes (VWR International, Mississauga, ON, Canada) were used for the experiments using plates. After plating the seeds or seedlings, the petri dishes were sealed with Parafilm (BEMIS, USA) to prevent contamination. Seedlings were considered germinated when the radical emerged from the seed coat. Therefore, day 1 of germinated seeds in all of the experiments refers to the day that the radical was visible from the seed coat. Plates (petri dishes) were kept at 4°C in the dark for 2 days and then placed in a growth chamber (Conviron, Winnipeg, MB, Canada) with a 16/8 h light/dark cycle, a photosynthetic photon flux density (PPFD) of 100  $\mu\text{mol (photons) m}^{-2} \text{ s}^{-1}$  and constant 22°C temperature. If seedlings required transfer to soil, 7 days after their germination on MS, they were transferred to square plastic 6 cm (100 mL volume) or 10 cm (350 mL volume) pots containing Sunshine Mix#1 Professional Growing Mix (Sun Gro Horticulture Inc, Vancouver, BC). The pots were placed under normal growth conditions (PPFD of 100  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ , 16/8 h light/dark cycle, 22°C temperature) or other specific conditions based on the specific experiment, described in sections 2.3.1 and 2.3.1. The described growth chamber conditions above are within the range of optimal growth conditions for Arabidopsis according to ABRC. Thus, in this thesis it is referred to as Arabidopsis standard growth condition.

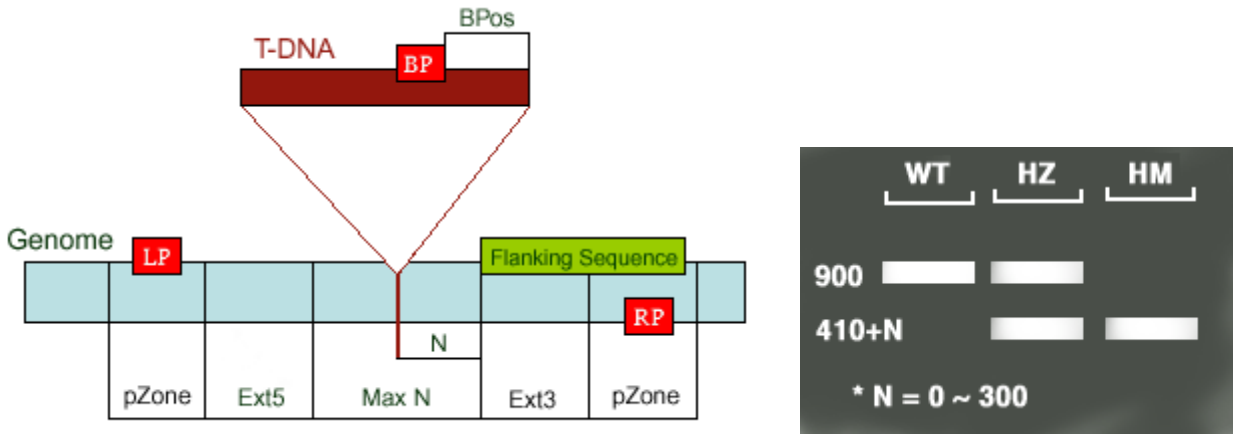
Rosette leaves were collected from fully expanded leaf tissue in the same developmental stage for each experiment. In early experiments, the length of the primary root of seedlings was measured using a ruler and later, photograph of plates were taken and the accuracy of measurements were checked with ImageJ (Schneider *et al.*, 2012; <http://imagej.nih.gov/ij/>). The fresh (FW) or dry weight (DW) of tissues or seedlings were measured with an auto-calibrating analytical balance (Mettler-Toledo GmbH, Switzerland). If the samples needed to be freeze dried, they were frozen, then placed in a freeze drier (Labconco Freezone, USA) overnight to remove water from the frozen samples under vacuum.

## **2.2. T-DNA insertion mutant lines**

T-DNA insertion mutants for sequences coding the desired ureide metabolism enzymes were identified using the T-DNA express Arabidopsis gene mapping tool (<http://signal.salk.edu/cgi-bin/tdnaexpress>). Seed stocks were obtained from the Arabidopsis Biological Resource Center (ABRC) for T-DNA insertions in the *XDHI* (At4g34890), *XDH2* (At4g34900), *UO* (At2g26230), *ALN* (At4g04955), *AAH* (At4g20070), *UGlyAH* (At5g17050) and *UAH* (At5g43600) genes. The T-DNA insertion mutant lines which were ordered from ABRC are listed in Table B1 (Appendix B).

### **2.2.1. PCR-based screen**

Homozygous lines for the T-DNA insert were screened by PCR using gene-specific and T-DNA-specific primers. The SALK T-DNA verification primer design program (<http://signal.salk.edu/tdnaprimers>) was used to pick left and right primers (LP and RP) for each line. For the wild-type allele, LP and RP primers were designed on flanking sequences, while for the mutant allele one primer was designed from flanking sequence and the other one was designed from T-DNA (Figure 2.1). The sequence of all primers designed with the T-DNA primer design program and used for screening the T-DNA insertion lines in this study are listed in Table B2 (Appendix B).



**Figure 2.1. PCR-based screening of T-DNA insertion mutants.**

N: the difference of the actual insertion site and the flanking sequence position, usually 0 - 300 bps; MaxN: maximum difference of the actual insertion site and the sequence, default 300 bps; pZone: regions used to pick up primers, default 100 bps; Ext5 and Ext3: regions between the MaxN to pZone, reserved not for picking up primers; LP: left, Right genomic primer; BP: T-DNA border primer; LB: the left T-DNA border primer. BPos: the distance from BP to the insertion site. The figure and the explanations are taken from: (<http://signal.salk.edu/tdnaprimers>).

In this research, the universal T-DNA primer LBb1.3 (ATTTTGCCGATTTTCGGAAC) was used to screen SALK lines and the LB3 primer (TAGCATCTGAATTTTCATAACCAATCTCGATACAC) was used to screen SAIL lines. As described in Figure 2.1, by using three primers (LBb1.3+LP+RP) for SALK lines, WT (wild-type - no insertion) should have a product of about 900-1100 bps (from LP to RP), for HM (homozygous lines - insertions in both chromosomes) a band of 410+N bps (from RP to insertion site 300+N bases, plus 110 bases from LBb1.3 to the left border of the vector), and for HZ (heterozygous lines - one of the pair of chromosomes with insertion) both bands could be detected. To detect homozygous lines, the LBb1.3 and LB3 primers were used along with genomic primers for each line (Table B2).

### **2.2.2. DNA extraction**

To screen the T-DNA insertion lines, DNA was extracted from leaf tissue using a modified method of Edwards *et al.* (1991). The DNA extraction buffer was: 250 mM NaCl (Millipore Ltd, Etobicoke, ON, Canada), 25 mM EDTA (Sigma, USA), 0.5 % (w/v) SDS (Sigma, St. Louis, MO, USA) and 200 mM Tris-HCl (pH 7.5) (VWR). All centrifugation was carried out at room temperature. For all of the non-refrigerated centrifugation of this research, an 18 micro-centrifuge (Beckman Coulter, ON, Canada) was used. The leaf tissue was ground in 1.5 mL micro-centrifuge tubes using 7 cm plastic tissue grind pestles (Fisher Scientific, MA, USA). To this, 500 µL of DNA extraction buffer was added and samples were centrifuged at 17,500 g for 5 min. Supernatant (350 µL) was transferred to fresh 1.5 mL micro-centrifuge tubes containing 350 µL isopropanol. The tubes were inverted a few times and then centrifuged for 10 min at 14,000 x g in a bench-top mini-centrifuge (ManSci Inc., Orlando, Florida). Afterwards, the supernatant was discarded and 100 µL sterile ddH<sub>2</sub>O was added to the pellet. The DNA pellet was re-suspend in the ddH<sub>2</sub>O by vortexing and shaking at room temperature.

### **2.2.3. PCR conditions**

For all PCRs and RT-PCRs, an Eppendorf thermal cycler (Eppendorf MasterCycle Gradient, Germany) was used. For all PCRs the TopTaq DNA Polymerase kit (Qiagen, Germany) was used. The final concentration of PCR reagents in the PCR was: 1X PCR buffer, 200 µM of each deoxynucleotide, 0.2 µM of each primer, 0.04 units/µL DNA polymerase and 1.5 mM MgCl<sub>2</sub>.

In order to identify homozygous, heterozygous or wild-type T-DNA insertion lines, PCRs with DNA samples were carried out with a 96 °C initial denaturation for 3 min, followed by 35 cycles consisting of, 94 °C 30 s, 60 °C 30 s, 72 °C 2 min, and a final extension at 72 °C for 4 min. PCR products were visualized on a 1.2% (w/v) agarose (BioShop) gel, run in TAE (1X) buffer and stained with ethidium bromide (EtBr; Sigma-Aldrich) using standard procedures (Sambrook and Russell, 2001). A PowerPac basic power supply (Bio-Rad, USA) at 60 volts for 45 min was used for gel electrophoresis and a BioDoc Transilluminator (UPV, USA) was used for gel imaging. A GeneRuler 1 kb DNA ladder (ThermoFisher Scientific Waltham, MA, USA) was used for sizing.

#### **2.2.4. Back cross**

The mutant that showed tolerant phenotype (*aln-3*), was backcrossed to Col-0 to reduce the possibility of the effect of extra T-DNA insertions in the genome. Crossing was done when the mother plants (*aln-3*) had 5-6 inflorescences and the pollen donors (Col-0) started to form siliques. First, the mature siliques and open flowers were removed from the mother plants using forceps. A magnifier with 10-20x magnification was used for the rest of the procedures. Dissecting forceps (Superfine Tip, Straight, 108 mm (4<sup>1</sup>/<sub>4</sub>" ) (VWR) were sterilized by 96% ethanol and used for crossing. All immature anthers were removed from the mother plants. Open and mature flowers from the father plant were used for pollination. Anthers with visible pollen were held with forceps and tapped on a recipient stigma. When the stigma was fully covered with pollen, the pollinated inflorescence was marked with a thread around the stem. The plants were returned to the growth chamber and were allowed to form mature siliques. Seeds were collected from the mature siliques and were planted to form mature T<sub>1</sub> plants. To identify plants that were heterozygous for *ALN*, leaf tissue of mature BC<sub>1</sub> plants was sampled for DNA extraction. PCR-based genotyping was done on mature plant leaves to select the desirable offspring genotype.

The *ALN* heterozygous plants generated from the first back cross (BC<sub>1</sub>) were backcrossed against Col-0 for a second time. As shown in Figure 2.2, half of the BC<sub>2</sub> generation was heterozygous and the other half was wild-type for *ALN*. The segregation ratio of 1/2 heterozygous and 1/2 wild-type indicated that the heterozygous seeds are the result of backcross and not self-fertilization. Again, the PCR-based screen was used to identify the plants with a heterozygous genotype for *ALN*. To obtain homozygous *aln* plants for the T-DNA insertion, the heterozygous BC<sub>2</sub> plants were allowed to self-fertilize. As predicted, the genotype of the BC<sub>2</sub>F<sub>1</sub> would be 1/4

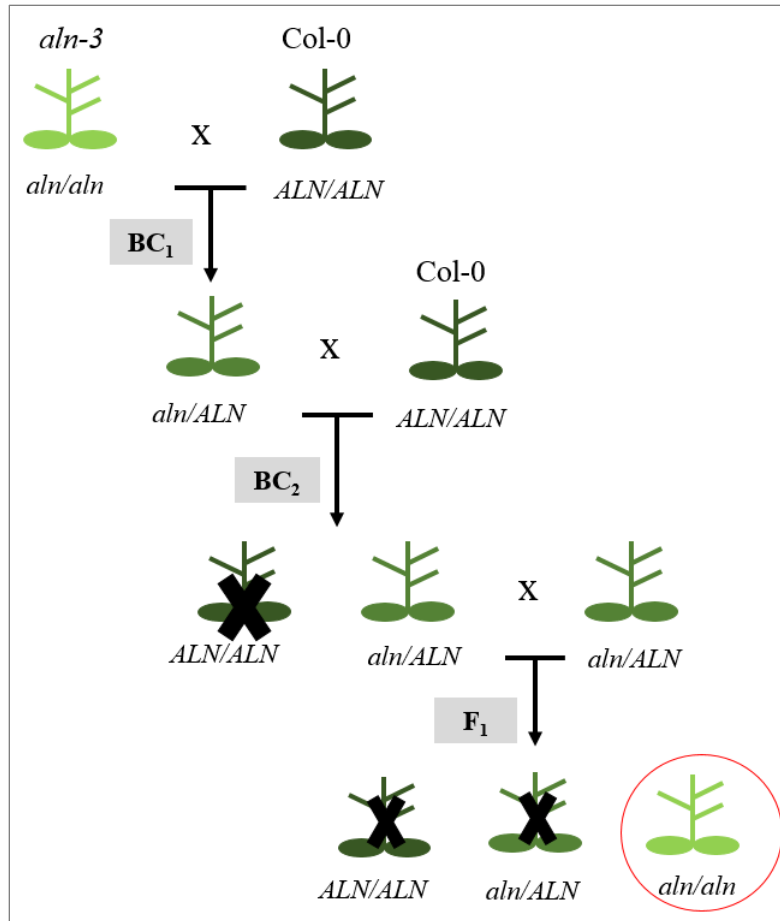
wild-type, 2/4 heterozygous and 1/4 homozygous. Plants homozygous for the insertion were identified by PCR and used for self-fertilization. Figure 2.2 represents the schematic procedures of the backcrosses of *aln-3* against Col-0. For experiments using the mutant, the BC<sub>2</sub>F<sub>3</sub> generation are used.

## **2.3. Abiotic stress conditions**

### **2.3.1. Drought**

When seedlings were 7-days old, they were transferred to pots and were allowed to grow under standard *Arabidopsis* growth conditions. When the plants were 4-weeks old, drought stress was imposed by withholding water for 12 days. On day 0 (the day before withholding water), pots were watered to saturation, then they were allowed to drain and weighed. Withholding water for 5, 10 and 12 days led to a 56, 77.5 and 78.5 % reduction in pot weight, respectively. Plants were re-watered after 12 days and their recovery was monitored. 20-20-20 Plant-Prod® fertilizer (200 mg L<sup>-1</sup>; Master Plant-Prod Inc, Ancaster, ON) was added to irrigation water weekly before the drought experiment. The FW and DW of the parts above ground (aerial parts of plants) were measured on the different time points as mentioned in the result section.

Soil gravimetric water content (SGWC) of plants under drought stress was measured as described by Gaxiola *et al.* (2001). Individual 6-cm pots with approximately 100 mL of soil, each contained a single plant and were weighted on day 0, 5, 10 and 12 after withholding water. At the end of drought stress treatment, the pots were dried in a 70 °C oven overnight and then weighed again. Afterwards, the weight of each pot was subtracted from the values measured on day 0, 5, 10 and 12. SGWC was calculated by dividing the soil water content at each time point by the oven dried pot weights.



**Figure 2.2. Backcrosses of *aln-3* against the Col-0.** BC<sub>1</sub>: First back cross generation; BC<sub>2</sub>: Second back cross generation. The first back cross results in all heterozygous genotype. The second back cross results in 1:1 ratio of heterozygous and wild-type genotype. The heterozygous BC<sub>2</sub> plants were allowed to self-fertilize. Therefore, in F<sub>1</sub>, 1/4 wild-type, 2/4 heterozygous and 1/4 homozygous (the ratio of 1:2:1) was generated. The homozygous F<sub>1</sub> plants were self-fertilized for further experiments.



### 2.3.2. Light treatments

When seedlings were 7-day-old, they were transferred to pots and allowed to grow for an additional 10 days under standard *Arabidopsis* growth condition. When plants were 17-day-old, pots were transferred to moderate or increased light condition growth chambers for an additional 21 days. Stress conditions were obtained using chambers (Convion) with a day/night temperature (22/10°C) and a light/dark cycle (21/3 h). The two different growth irradiance (determined at soil level) were used: moderate-light and increased-light which corresponded to PPFDs of 250 and 750  $\mu\text{mol m}^{-2} \text{s}^{-1}$  respectively (Malik *et al.*, 2016). Plants were irrigated with 200 mg L<sup>-1</sup> of 20-20-20 Plant-Prod® fertilizer (Master Plant-Prod Inc) once a week.

### 2.3.3. NaCl and mannitol treatments

To examine ureide concentration and gene expression in wild-type seedlings in response to salinity and osmotic stresses, NaCl and mannitol were used, respectively. Col-0 seeds were first germinated on MS plus sucrose (1%). Three days after germination, seedlings were transferred to MS media without sucrose containing 200 mM NaCl (OmniPur, EMD Millipore Canada, Etobicoke, Canada) or 250 mM mannitol (USP grade, AMRESCO, Solon, OH, USA) as well as control media without NaCl, mannitol and sucrose. Sucrose was removed from MS plates during NaCl and mannitol treatments to eliminate the effect of sucrose on seedlings growth and development. To detect the effect of exposing seedlings to NaCl and mannitol on ureide accumulation and gene expression, the whole seedlings were sampled at different time points starting with three days after germination (before stress treatment), then every two hours for the first 10 hours after shifting to NaCl or mannitol. The seedlings were also sampled 4 days after exposure to NaCl or mannitol stress. After four days, the seedlings usually did not survive under each stress treatment.

### 2.3.4. NaCl tolerance test

To test the effect of NaCl on germination, Col-0 and *aln-3* seeds were germinated on MS media (without sucrose) supplemented with 0, 50, 75 and 100 mM NaCl. The number of germinated seeds was counted 3 days after transferring to germination permissive conditions (as described in section 2.1). Root length was measured and the number of seedlings that developed true leaves was counted seven days after seed germination.

To test the effect of stress tolerance when Col-0 and *aln-3* are shifted to NaCl media, the Col-0 and *aln-3* seeds were first germinated on MS media plus sucrose for 5 days. The 5 days old seedlings were transferred to media supplemented with 100 mM NaCl as well as 0 mM NaCl as control (both without sucrose) (Quan *et al.*, 2007). Fresh weight and root length of seedlings were measured 9 days after shifting to the new media.

### **2.3.5. NaCl and exogenous allantoin tolerance test**

To assess the effect of exogenous allantoin application on Col-0 germinated under NaCl stress, Col-0 seeds were sown on MS control, 100 mM NaCl, 100 mM NaCl + 0.1 mM allantoin, 100 mM NaCl + 1 mM allantoin, 100 mM NaCl + 0.1 mM proline and 100 mM NaCl + 1 mM proline media. No sucrose was added to these media. Fresh weight and root length of seedlings were measured 7 and 10 days after germination. The proline used in these experiments was L-proline (BDH chemicals, UK).

To assess the effect of exogenous allantoin application on NaCl stress tolerance of Col-0 seedlings, Col-0 seeds were first germinated on MS media containing 1% sucrose. 5 days after germination, seedlings were transferred to MS control, 100 mM NaCl, 100 mM NaCl + 0.1 mM allantoin, 100 mM NaCl + 1 mM allantoin, 100 mM NaCl + 0.1 mM proline and 100 mM NaCl + 1 mM proline media. None of these media contained sucrose. Seedlings were grown for 9 additional days in these media, after which fresh weight and root length of 2-week-old seedlings were measured.

### **2.4. Quantification of chlorophyll and anthocyanin**

Chlorophyll was extracted from rosette leaves or whole seedlings with 80% (v/v) acetone (HPLC grade, Fisher Chemical, PA, USA). Absorption was measured spectrophotometrically at 663 and 645 nm according to Lichtenthaler and Wellburn (1983). Chlorophyll amount was determined as µg per mL which was then normalized based on the fresh weight of the extracted tissue and presented as µg per mg (or mg per g).

Anthocyanin extraction was based on the method of Lange *et al.* (1970) with minor modifications. The rosette leaf tissue was weighed and placed in 1.5 mL micro-centrifuge tubes containing 1 mL extraction buffer of 18% (v/v) 1-propanol (HPLC grade, EMD Chemicals, ON, Canada), 1% HCl (Fisher Chemicals) and 81% water. The modifications were according to Noh

and Spalding (1998) to detect the anthocyanin level in Arabidopsis. Leaf samples in tubes were ground using plastic tissue grinding pestles and then tubes were placed in boiling water for 3 min. Tubes were incubated in the dark and at room temperature for 2 h and then centrifuged at 18,000 *g* for 2 min. The amount of total anthocyanins in the supernatant was quantified spectrophotometrically. Values was calculated as  $A_{535} - 2.2 (A_{650})$  (Lange *et al.*, 1970).

## 2.5. Photochemical efficiency of PSII

The photochemical efficiency of PSII ( $F_v/F_m$ ) of rosette leaves attached to the plants in the pots was measured at room temperature. A PAM 2000 chlorophyll fluorometer (WALZ, Germany) or a Dual-PAM-100 measuring system (WALZ, Germany) were used to detect  $F_v/F_m$  for control and drought- or light-stressed plants. The  $F_v/F_m$  ratio of all samples was calculated as  $(F_m - F_o)/F_m$  after 15 min dark-adaption.

## 2.6. Staining and quantification for $O_2^-$ and $H_2O_2$

Histochemical staining for  $O_2^-$  and  $H_2O_2$  was performed with nitroblue tetrazolium (NBT; BioShop) and diaminobenzidine (DAB; Gold Biotechnology, St. Louis, MO, USA), respectively, as previously described by Brychkova *et al.* (2008) with minor modifications. To detect  $O_2^-$ , rosette leaves were detached and floated in 0.8 mM NBT for 2 h in the dark. For  $H_2O_2$  detection, rosette leaves were placed in 1 mg mL<sup>-1</sup> DAB solution (pH 5.0) for 4 h in the light. Both solutions were made in sterile ddH<sub>2</sub>O. After staining times, the solutions were poured away and stained leaves were rinsed in 96% ethanol and boiled for 10 min to remove chlorophyll. Ethanol was removed and the samples rinsed with ddH<sub>2</sub>O. The levels of  $O_2^-$  and  $H_2O_2$  were visualized as dark-blue and brown coloration respectively and photographed.

Quantification of  $O_2^-$  and  $H_2O_2$  was performed using the method described by Ramel *et al.* (2009). Leaf samples were weighed and stained with NBT and DAB as described previously. NBT- and DAB-stained leaves were immediately ground in liquid nitrogen. To quantify  $O_2^-$ , KOH-DMSO solution (1:1.16 v/v; 2 M KOH:DMSO) (Fisher Chemical) was added to the leaf powder. Samples were centrifuged for 10 min at 9,000 *g* and absorption was measured at 630 nm. For quantification of  $H_2O_2$ , 0.2 M HClO<sub>4</sub> (Anachemia, QC, Canada) was applied to the leaf powder and centrifuged for 10 min. The  $A_{450}$  of the extracts was measured and compared with the standard

curve. The standard curve for  $O_2^-$  was obtained from known amounts of NBT in 2 M KOH-DMSO. To obtain the  $H_2O_2$  standard curve, known concentrations of  $H_2O_2$  (35%, Anachemia) in 0.2 M  $HClO_4$ -DAB was used. Serial dilutions of 15  $\mu$ M to 0.5  $\mu$ M for NBT and 100  $\mu$ M to 1  $\mu$ M  $H_2O_2$  were used to graph the standard curves. Standard curves with  $R^2$  value of  $> 0.996$  were generated and used to quantify samples.  $O_2^-$  levels are represented as reduced NBT since NBT is reduced and forms blue color by reaction with  $O_2^-$ . The average rate that NBT is reduced by  $O_2^-$  is based on an extinction coefficient:  $(5.88 \pm 0.12) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  (Blelski *et al.*, 1980).

## 2.7. Sample preparation for analysis by HPLC

Both standards and plant samples were prepared in a solution of ddH<sub>2</sub>O as solvent. To prepare the standards, known concentrations of uric acid (99%; Alfa Aesar, Ward Hill, MA, USA), allantoin (Sigma-Aldrich) and allantoate (Sigma-Aldrich) were used. To obtain the standard curves of ureide compounds, serial dilutions of each compound in ddH<sub>2</sub>O were prepared. Dilution series of 50 nmol  $\mu$ L<sup>-1</sup> to 0.05 nmol  $\mu$ L<sup>-1</sup> for uric acid; 50 nmol  $\mu$ L<sup>-1</sup> to 0.1 nmol  $\mu$ L<sup>-1</sup> for allantoin and 5 nmol  $\mu$ L<sup>-1</sup> to 0.01 nmol  $\mu$ L<sup>-1</sup> for allantoate were used. Standard curves were graphed and in each case resulted in an  $R^2$  value of  $> 0.998$  (Figure C1; Appendix C). Plant tissue or whole seedlings sample were ground in liquid nitrogen using a mortar and pestle. For mature plant tissue, 1 mL of ddH<sub>2</sub>O and for seedlings, 0.5 mL ddH<sub>2</sub>O was added to the ground powder. Their extracts were homogenized in sterile ddH<sub>2</sub>O, they were centrifuged at 15,000 g for 10 min, at 4 °C, in a refrigerated centrifuge (Beckman Coulter Microfuge 22R, USA) to collect the pellet. The supernatant was filtered through a 0.2  $\mu$ m syringe-filter (13 mm; VWR) and then placed in the high performance liquid chromatography (HPLC) system for analysis.

### 2.7.1. HPLC parameters

The levels of uric acid, allantoin and allantoate were determined by HPLC using an Agilent 1200 series HPLC system (Agilent Technologies Inc, Mississauga, ON, Canada). For the separation of these compounds, an organic acid column (Aminex® HPX-87H- 300 x 7.8 mm- Bio-Rad Laboratories, Hercules, CA, USA) was used. The mobile phase of 2.5 mM  $H_2SO_4$  (pH~3.0) (EMD Inc. ON, Canada) was first filtered through ZAPCAP-CR bottle top filters (0.2  $\mu$ m pore size, Whatman, NJ, USA) and then applied to the system to pass through the degasser before

reaching the pump and column. Samples were transferred to the HPLC in 2 mL wide Opening (9 mm) Amber Glass Screw Top Vials (Agilent Technologies Inc, CA, USA) that were kept at 4 °C in the auto-sampler until injection. Flow rate was 0.5 mL min<sup>-1</sup>, the column temperature was 20°C and the injection volume was between 10-50 µL, depending on the sample. Total run time with this method was 45 min and compounds were detected at 190 nm using a diode-array detector. Three ureide compounds were detected using the standard retention time for each compound; uric acid, allantoin and allantoate at approximately 19, 22 and 24 min, respectively (Figure C2; Appendix C).

## **2.8. Gene expression analysis**

The sequences of ureide metabolic genes were obtained from Phytozome (<http://phytozome.jgi.doe.gov>). Phytozome is a portal of plant comparative genomics of the Energy's Joint Genome Institute department (<http://phytozome.jgi.doe.gov>) and it provides access to the sequenced *Arabidopsis thaliana* genome. To analyse the expression of genes coding for ureide enzymes, gene-specific primers were designed using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) software (Koressaar and Remm, 2007; Untergrasser *et al.*, 2012). When primers were designed, their sequence and amplification region was double checked in the Primer-BLAST tool at the National Center for Biotechnology Information (NCBI; Ye *et al.*, 2012).

Table B3 (Appendix B) shows the primers that were used to test the effect of T-DNA insertion on expression of mutant genes in the homozygous T-DNA insertion lines. Table B4 (Appendix B) shows the primers used to analyze the expression of ureide genes under abiotic stress conditions.

### **2.8.1. RNA extraction and cDNA synthesis**

Total RNA was isolated from rosette leaf tissue or from whole seedlings using the E.Z.N.A Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA). Samples stored at -80 °C after freezing in liquid nitrogen were used for RNA extraction. All centrifugations were done at room temperature. Approximately 25 mg (fresh weight) of leaf tissue and 7 mg (fresh weight) of seedlings were used for RNA extractions. Frozen tissue was ground under liquid nitrogen using a mortar and pestle and immediately 500 µL extraction buffer/ β-mercaptoethanol (extraction buffer: Supplied within the

kit;  $\beta$ -mercaptoethanol: EMD Chemicals) was added to the grounded tissue. Tissue was then collected in a 1.5 mL micro-centrifuge and the RNA extraction procedures was done according to manufacture protocol.

RNA concentration was quantified by spectrophotometric measurement using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). All RNA samples were stored at -80 °C or used for making cDNA immediately. One  $\mu$ g of total RNA was used to synthesize the first strand of cDNA using QuantiTect® Reverse Transcription Kit (Qiagen, Germany) according to the manufacture's protocol.

### **2.8.2. Reverse transcriptase PCR**

For light treated samples, RT-PCR was carried out with a 95 °C initial denaturation for 3 min, followed by 32 cycles consisting of, 94 °C 30 s, 60 °C 40 s, 72 °C 1 min, and a final extension at 72 °C for 7 min. For all the other experiments, RT-PCR conditions were: 95 °C initial denaturation for 3 min, followed by 32 cycles consisting of, 94 °C 30 s, 60 °C 35 s, 72 °C 1 min, and a final extension at 72 °C for 4 min. TopTaq DNA Polymerase kit (Qiagen, Germany) was used in all RT-PCR amplifications. The final concentrations of reagents and reaction volume were similar to those in section 2.2.3 and 1  $\mu$ L cDNA was used in each 25  $\mu$ L PCR reaction. *ACTIN7* (At5g09810) was used as a reference gene. A 1 kb DNA Marker GeneRuler (Real Biotech Corporation; Taiwan) was used for sizing of amplifications of stress treatment experiments. .

### **2.8.3. Quantitative reverse transcriptase PCR**

For quantitative reverse transcriptase PCR (qRT-PCR), primers designed by Brychkova *et al.* (2008) were used (Table B5; Appendix B). Quantitative RT-PCR amplification was performed as described by Brychkova *et al.* (2007) in 40 cycles, consisting of an initial denaturation of 95 °C for 3 min, followed by 20 s at 95 °C, 20 s at 65 °C and 30 s at 72 °C. The reactions were done in a final of 25  $\mu$ L using EvaGreen Dye 20X (Biotium, Hayward, CA, USA) with the similar final concentrations and reaction volume as described in section 2.2.3. For EvaGreen Dye 20X, the final concentration of 1X was used.

Fluorescence increments of each reaction were simultaneously monitored using the iCycler iQ5 Multicolor real time PCR detection system (Bio-Rad). In the real time PCR detection system, 96 well flat top clear PCR microplates (Axygen, CA, USA) were used. PCR amplification efficiency rate was tested for all primers by the standard curve method (Livak, 2001). The real time PCR standard curve represents the semi log regression line plot of the  $C_t$  (threshold cycle) values vs. log of the concentration of samples. The standard curve slope of  $-3.32$  ( $y = -3.32x$ ) indicates 100% PCR efficiency (Livak, 2001). To analyze PCR efficiency, a dilution series of a cDNA sample was prepared and then real time PCRs were performed for each primer set using the cDNA dilution series. The slope of the standard curve for each primer was measured using  $C_t$  data of the serial dilution. When PCR efficiency of  $> 99\%$  was obtained for each primer, the expression fold change of *UO* and *ALN* under drought and control (watered) condition in leaves was determined. All cDNA samples were analyzed in triplicate, and each cDNA was derived from three sets of independent experiments. Relative expressions of *UO* and *ALN* were calculated using the relative  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001). The Livak and Schmittgen (2001) method calculates expression fold change using these formula:

$$\Delta C_{t_{\text{target}}} = C_{t_{\text{control}}} - C_{t_{\text{treatment}}}$$

$$\Delta C_{t_{\text{reference}}} = C_{t_{\text{control}}} - C_{t_{\text{treatment}}}$$

$$\Delta\Delta C_t = \Delta C_{t_{\text{reference}}} - \Delta C_{t_{\text{target}}}$$

The fold change in expression of a target gene =  $2^{-\Delta\Delta C_t}$

In this experiment, *UBQ10* (At4g05320) and *ACTIN2* (At3g18780) were used as reference genes and all reactions were normalized with them. The reference genes revealed similar up or down-regulation trends and thus only the results based on *UBQ10* were presented. *UBQ10* demonstrated less variation under the experimental conditions and is also the reference gene used by Brychkova *et al.* (2008), allowing for more direct comparison. The relative transcript abundance on day 0 was set to 1 to which the untreated and treated samples following withholding of water was compared.

## 2.9. Statistical analysis

All experiments were repeated at least three times in independent experiments ( $n$ = number of independent replications). Student t-test and analysis of variance (ANOVA) test were performed to compare mean values using SPSS software Ver. 22.0 (IBM Statistical Product and Service Solutions), licensed to University of Saskatchewan (IBM, Markham, CA). Tukey HSD (honest significant difference) test from the same software was used to find means that are significantly different from each other in multiple treatment conditions. Differences at  $p < 0.05$  were considered significant. When no significant difference was found, statistical analysis are not shown. All the data are presented as means plus/minus the standard error of the mean (SEM). All graphs were prepared in Excel of Microsoft Office software (Microsoft Company; Mississauga, CA). Image editing for production of figures was done using GIMP 2.8.14 software ([www.gimp.org](http://www.gimp.org)).



## **CHAPTER 3. RESULTS**

### **3.1. T-DNA insertion mutants**

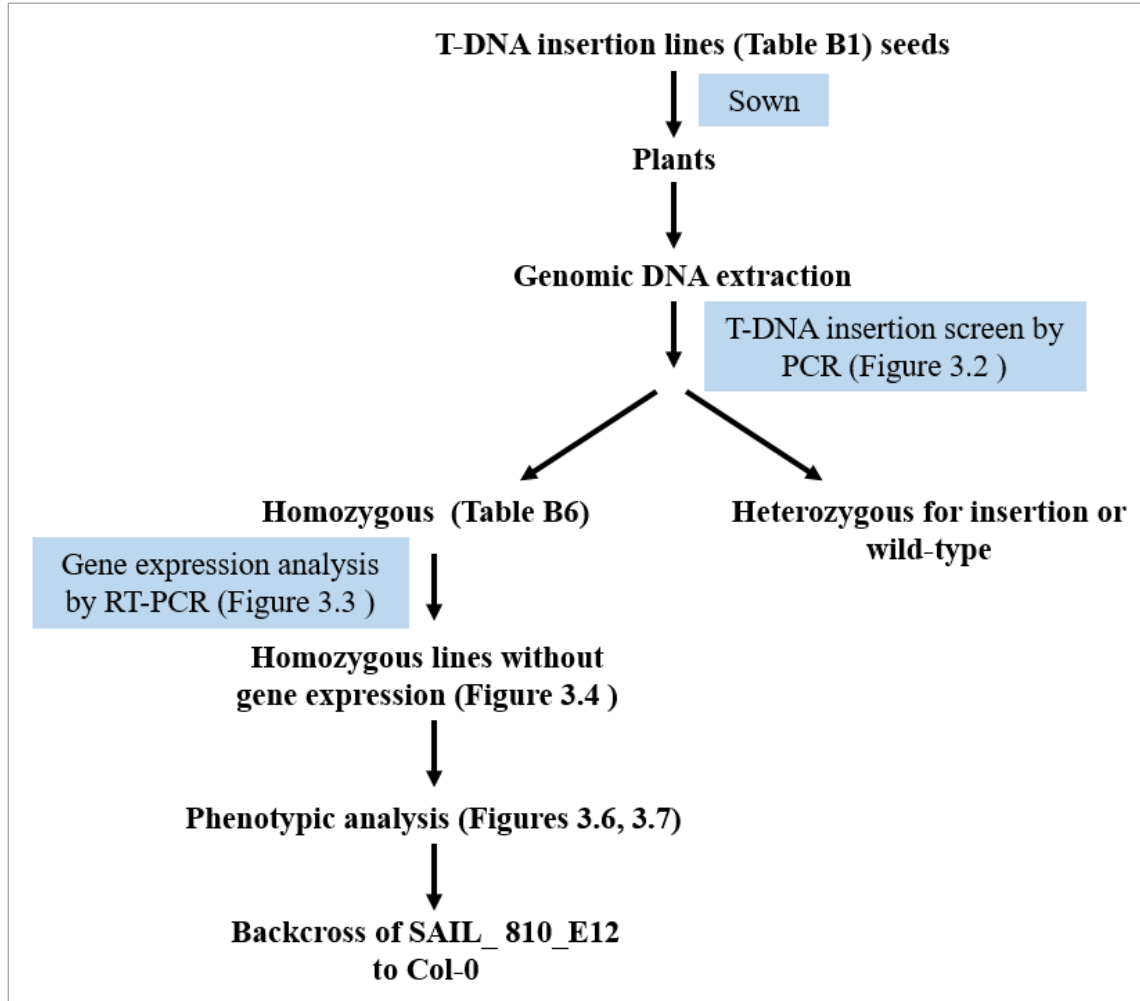
T-DNA insertion seed stocks were obtained from the Arabidopsis Biological Resource Center (ABRC) and listed in Table B1 (Appendix B). Figure 3.1 shows the screening procedures for the T-DNA insertion lines.

#### **3.1.1. Characterization of homozygous mutants**

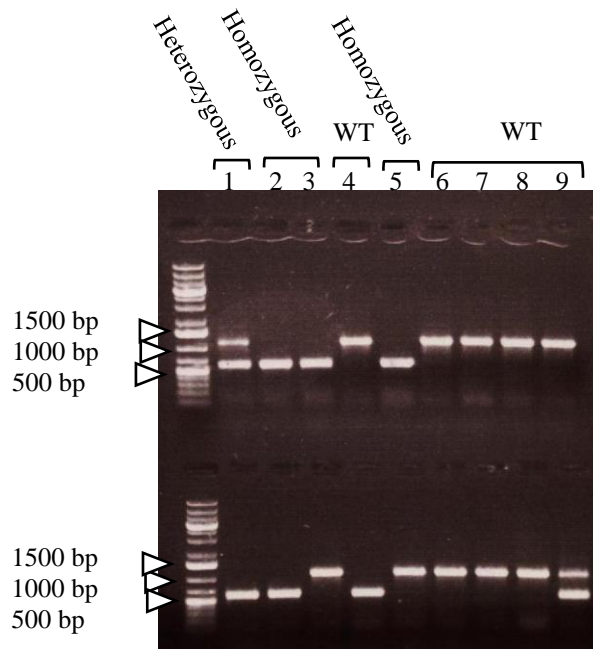
Screening of T-DNA insertion lines was done by PCR amplification using T-DNA primer and gene-specific primers (forward and reverse primers) as described in materials and methods. Figure 3.2 shows a representative gel of screening T-DNA insertion mutant for homozygous insertion.

#### **3.1.2. Gene expression analysis**

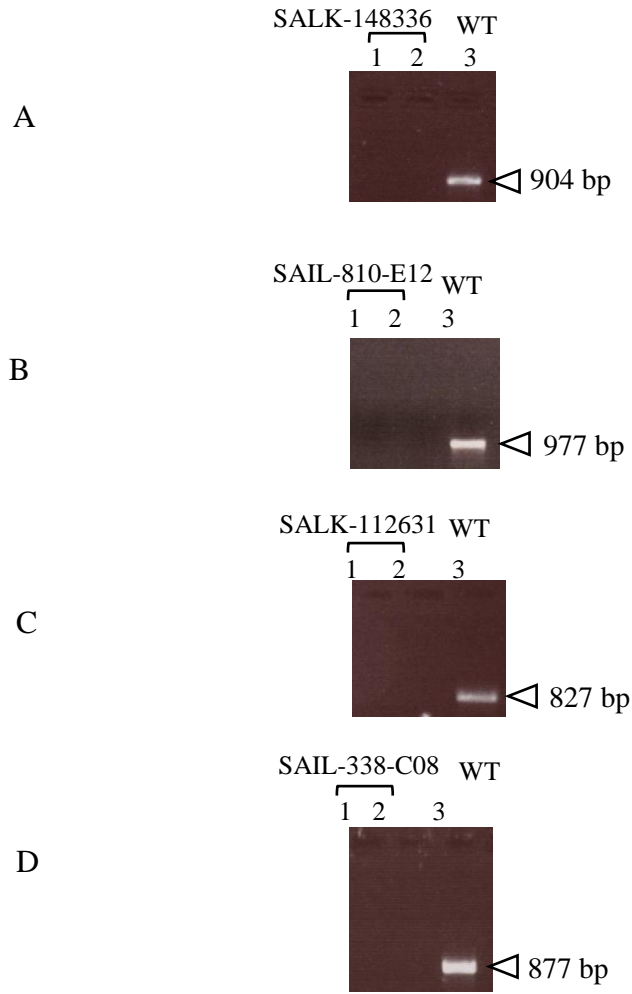
Overall, 12 homozygous lines were identified by PCR analysis. Expression of the gene of interest was tested by RT-PCR. Of those, four homozygous lines showed no expression of the gene as shown in Figure 3.3. The control RT-PCR for the four homozygous lines without gene expression was done using primers amplifying *ACTIN7* and is shown in Figure C3 (Appendix C). The list of 12 homozygous mutants and the effect of T-DNA insertion in the expression of the impaired gene is shown in Table B6 (Appendix B). Figure 3.4 shows the four homozygous mutants and the site of T-DNA insertion as well as the primer locations. Figure 3.5 shows the location of the four homozygous mutants in the ureide metabolism pathway.



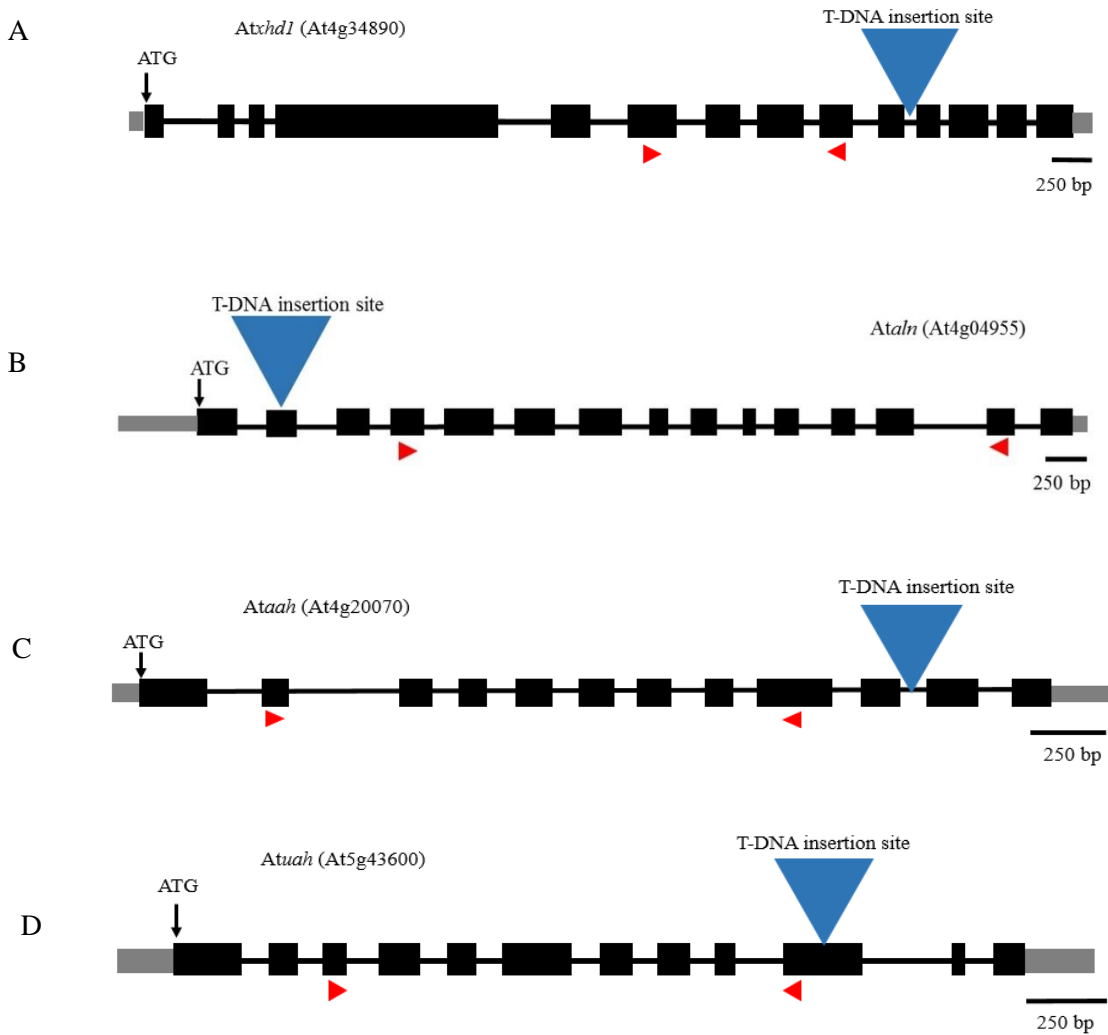
**Figure 3.1. The screening procedures of T-DNA insertion mutant lines**



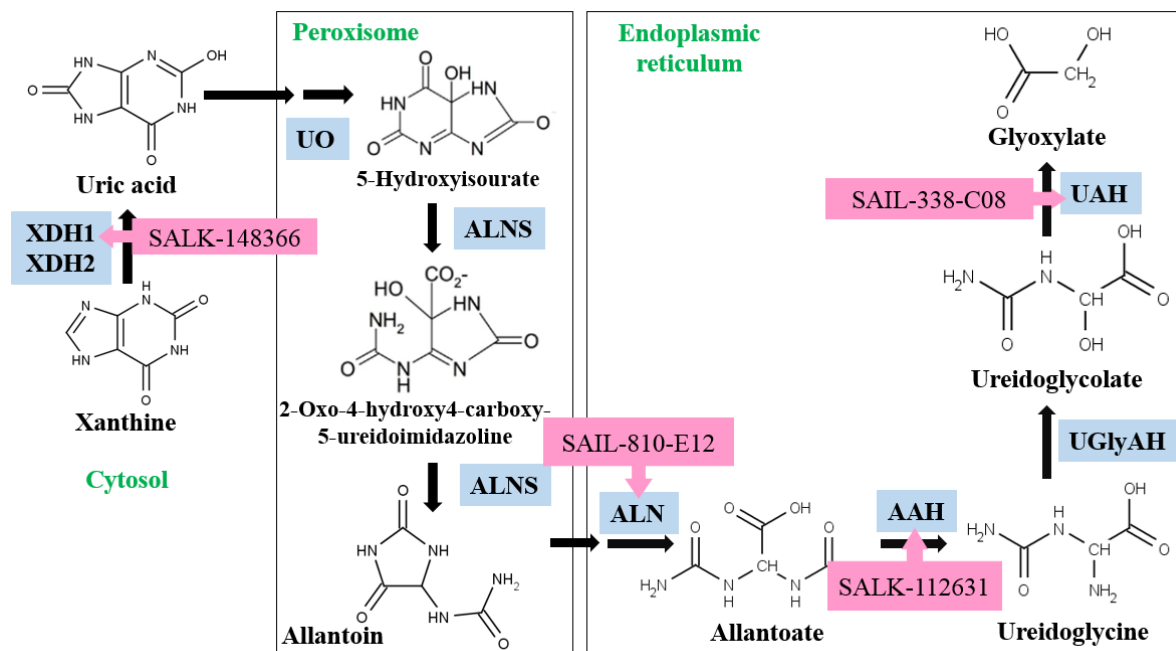
**Figure 3.2. An example image representing the genotyping of a T-DNA insertion mutant line.** The gel shows the PCR-based screening of SALK-148366 individual plants to identify homozygous plants using LP, RP and LB1.3 primers. As described in Figure 2.1, using three primers (LBb1.3+LP+RP), in the wild-type individuals (WT- no insertion) will result in a product of about 900-1100 bps (from LP to RP). Homozygous individual (insertions in both chromosomes) plants will have a product of 410+N bps (RP to insertion site 300+N bases + 110 bases from LBb1.3 to the left border of the vector). Heterozygous individuals (insertion in one chromosome) will have both bands.



**Figure 3.3. RT-PCR analysis to detect the effect of T-DNA insertion in the gene on gene expression of the four homozygous mutants.** Each number (1 or 2) for T-DNA insertion mutants, represents an individual homozygous plant grown from the individual seed from the initial T-DNA seed stock. Wild-type (WT) was used as control. (A) No *XDHI* gene expression for SALK-148336. (B) No *ALN* expression for SAIL-810-E12. (C) No *AAH* expression for SALK-112631. (D) No *UAH* expression for SAIL-338-C08. The sequence of primers used for RT-PCR-based gene expression and the size of each PCR product is listed in Table B3 (Appendix B).



**Figure 3.4. T-DNA insertions in Arabidopsis *XDHI*, *ALN*, *AAH* and *UAH* genes in the mutants.** (A) SALK-148366 line; *Atxhd1* (At4g34890, *XDHI*). (B) SAIL-810-E12 line; *Ataln* (At4g04955, *ALN*). (C) SALK-112631 line, *Ataah* (At4g20070, *AAH*). (D) SAIL-338-C08 line, *Atuah* (At5g43600, *UAH*). Black arrows indicate the translation start codon for each gene. Black boxes represent the exons, and the lines between filled black boxes represent introns. The gray boxes at the start and end of genes are 5'UTR and 3' UTR, respectively. The blue triangle shows the T-DNA insertion site in each gene. Red arrowheads indicate approximate location of primers used for RT-PCR-based gene expression (Table B3; Appendix B).



**Figure 3.5. Ureide metabolism genes with T-DNA insertions resulting in loss of transcript.** The pink callouts show each T-DNA mutant line and the arrow on each callout points to the impaired enzyme. XDH1, xanthine dehydrogenase 1 (SALK-148366, *Atxdh1*); ALN, allantoinase (SALK-810-E12; *Ataln*); AAH, allantoate amidohydrolase (SALK-112631, *Ataah*); UAH, ureidoglycolate amidohydrolase (SAIL-338-C08, *Atuah*)

### 3.1.3. Preliminary phenotypic tests

For the preliminary phenotypic tests, plants were treated with moderate- and increased-light. For this purpose, when plants were 17-day-old, they were shifted to chambers with moderate light ( $250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) or increased light ( $750 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) intensities, with long day photoperiod (day/night temperature  $22/10^\circ\text{C}$  and a light/dark cycle of 21/3 h). The phenotype of the four T-DNA insertion mutant lines were compared visually to wild-type plants under light treatments. Under the increased light condition, *aln* plants showed visually more tolerance compared to the other mutant lines and the Col-0 plants (Figure 3.6). Plants generally started to show morphological changes such as thickened leaves, reduced chlorophyll and enhanced levels of anthocyanin (purple leaf colour) after approximately 7 to 10 days under increased light condition. However, Col-0, *xdh1*, *aah* and *uah* plants turned purple earlier compared to *aln* plants (Figure 3.6 A). During the third week of increased light treatment, Col-0 and the other three mutant plants were completely purple and dried whereas *aln* plants still had some green tissue left (Figure 3.6 B). Figure 3.7 shows the same lines under moderate light conditions on the same time points. Visually, *aln* plants did not appear different from Col-0 plants in response to moderate light. The only main visual phenotype difference was seen for *xdh1* plants, which were visibly smaller compared to the Col-0. Delayed development and premature senescence phenotypes have already been reported for the mutants impaired in the expression of *XDHI* (Brychkova *et al.*, 2008; Watanabe *et al.*, 2010).

Based on the results from light treatments as well as additional experiments on Col-0 and the mutant plants under drought, or on seedlings under NaCl stress (Figure C4 and C5. Appendix C), it was concluded that *aln* mutants show more tolerance (for example as seen by more green pigment preservation under increased light) under abiotic stress compared to the other mutants and Col-0. Therefore, considering these observations and the higher concentration of allantoin under abiotic stress in the Col-0 (sections 3.2 and 3.3 of the results), the *aln* mutant was selected for back-crossing to Col-0 and additional experiments. The *aln* mutant in the present research was first isolated and referred to as *Ataln* by Werner *et al.* (2008). However, to date, no report on abiotic stress response of this line is available. Thus, in the present thesis, this line is referred to as *aln-3* in agreement with the nomenclature of the other two *aln* mutant lines, *aln-1* and *aln-2*, by Watanabe *et al.* (2014a).

The population of T-DNA insertion mutants might carry more than one T-DNA insertion in which case the phenotype of interest may be affected (Weigel and Glazebrook, 2008). Based on the information on Salk Institute Genomic Analysis Laboratory website, “approximately 50% of the lines contain a single insert, the other 50% of lines contain two or more inserts” (<http://signal.salk.edu>). Backcrossing the mutant to wild-type parents may remove any extraneous insertion and decrease the possibility of undesirable extra mutations (Weigel and Glazebrook, 2008). Here, the *aln-3* mutant was backcrossed against Col-0 two times as described in the chapter 2 and the BC<sub>2</sub>F<sub>3</sub> generation of *aln-3* was used for further experiments.



A



Col-0

*aln**aah**xdh1*

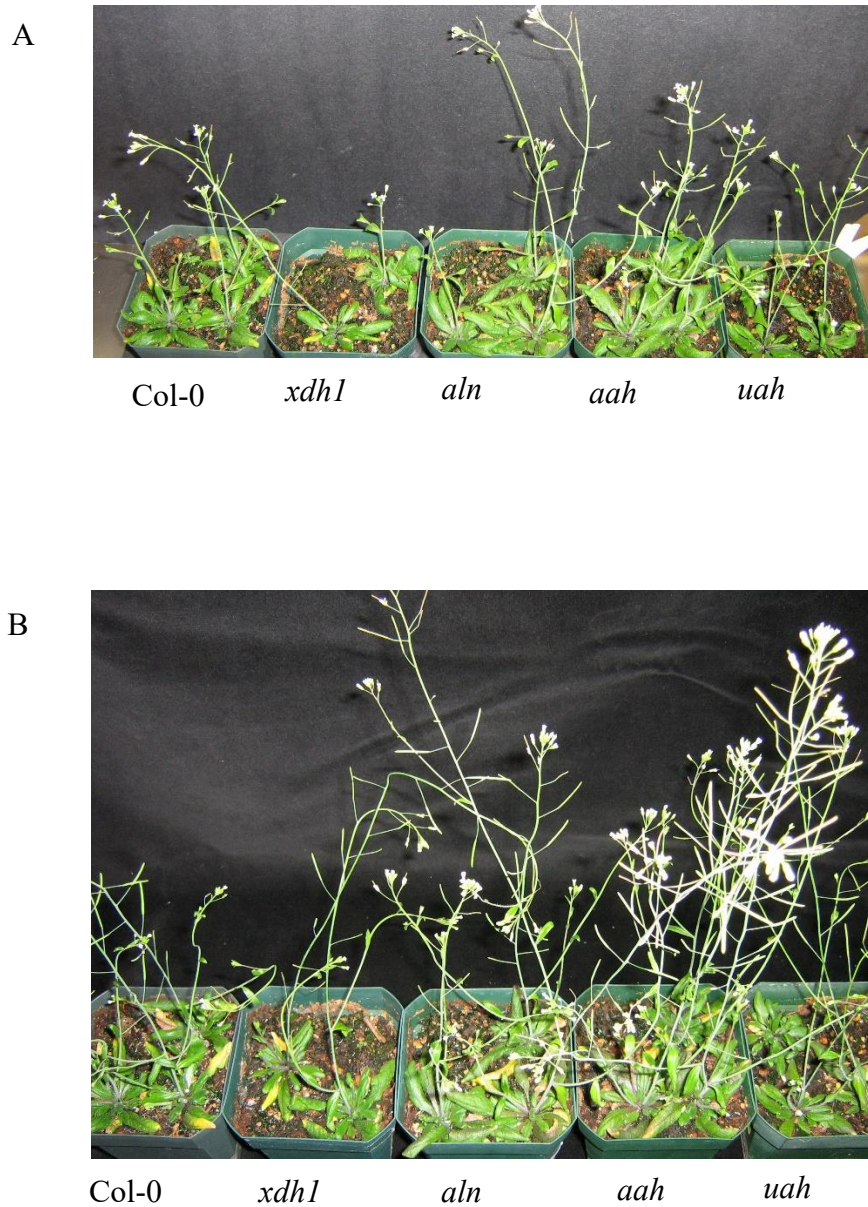
B



Col-0

*aah**uah**aln*

**Figure 3.6. Plant phenotypes under increased light condition.** When Col-0, *xdh1*, *aln*, *aah* and *uah* plants were 17-day-old, they were transferred to a chamber with increased light conditions. (A) Phenotype of plants when they were treated with increased light for 10 days. (B) Phenotype of plants when they were treated with increased light for 15 days.



**Figure 3.7. Plant phenotypes under moderate light condition.** When Col-0, *xdh1*, *aln*, *aah* and *uah* plants were 17-day-old, they were transferred to a chamber with moderate light conditions. (A) Phenotype of plants treated under moderate light intensity for 10 days. (B) Phenotype of plants when they were treated under moderate light intensity for 15 days.

## 3.2. Drought stress

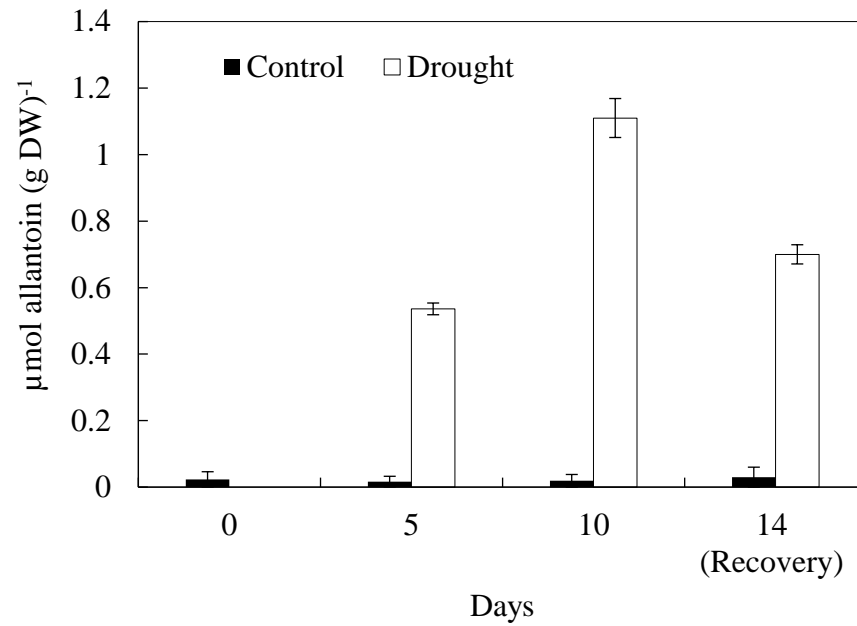
### 3.2.1. Ureide concentration in wild-type leaves

Uric acid, allantoin and allantoate were quantified by HPLC in rosette leaves of 4-week-old wild-type *Arabidopsis* plants under normal and drought stress conditions. Following drought stress, allantoin levels increased by day 5, reaching a maximum of  $1.1 \mu\text{mol (g DW)}^{-1}$  by day 10. After 12 days of drought stress, plants were re-watered and allantoin content was reduced to  $0.7 \mu\text{mol (g DW)}^{-1}$  on day 14 (2 days after re-watering) (Figure 3.8). Minor amounts of allantoin ( $< 0.09 \mu\text{mol (g DW)}^{-1}$ ) were observed in samples from plants in non-stress conditions which were tested at the same time points. Uric acid levels varied throughout the time period tested, from 0.05 to  $0.22 \mu\text{mol (g DW)}^{-1}$ , but did not respond to water limitation (Figure C6; Appendix C).

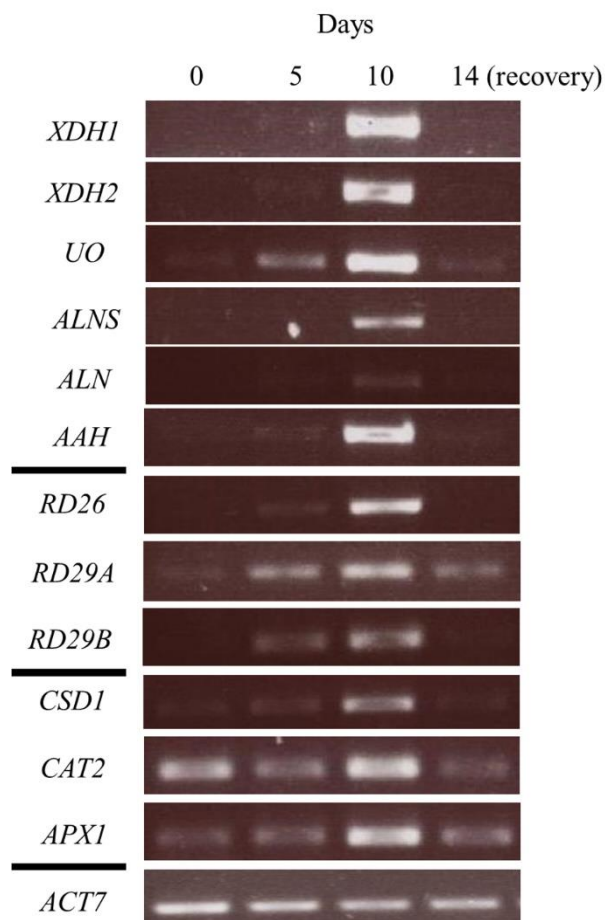
### 3.2.2. Expression analysis of ureide genes in wild-type leaves

Expression of six genes involved in ureide metabolism (*XDH1*, *XDH2*, *UO*, *ALNS*, *ALN*, *AAH*) were analyzed in Col-0 leaves by RT-PCR (Figure 3.9). Leaf samples were collected on day 0, 5, 10, and 2 days after re-watering, on day 14. After 10 days of withholding water, the expression of majority of ureide metabolic genes were induced, pointing to coordinated regulation of the pathway. Relative expression of *UO* showed an earlier increase, five days after withholding water. Interestingly, *ALN* was not as strongly upregulated compared to the other genes. Transcript levels of all genes, including *UO*, was clearly diminished after re-watering. The stress inducible genes, *RD29A*, *RD29B* and *RD26* used as drought markers (Yamaguchi-Shinozaki *et al.*, 1992, Yamaguchi-Shinozaki and Shinozaki, 1993; 1994) were induced by drought conditions, confirming the efficiency of the drought stress treatment. Expression of several genes encoding antioxidants, including *CSD1* (Cu/Zn SOD), *CAT2* and *APX1* were examined during drought stress. The induction of expression of these genes on day 10 was similar to the expression of most of ureide genes, increasing to a maximum on day 10 of drought and declining after re-watering the plants (Figure 3.9). The expression of ureide genes under well-watered conditions did not show any specific pattern of up or down regulation during the same time points (Figure C7; Appendix C).

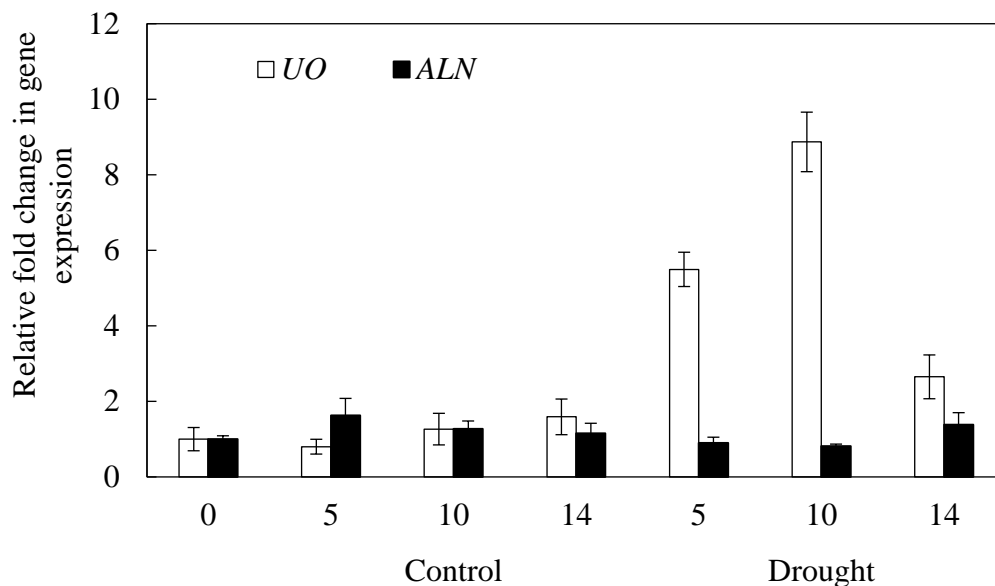
Based on their dissimilar expression patterns, I analyzed the change in expression of *UO* and *ALN* by qRT-PCR (Figure 3.10). No increase in expression of *UO* or *ALN* was observed in well-watered control plants when analyzed by ANOVA. Transcript of *UO* increased after 5 (> 5-fold induction) and 10 days (> 8.5-fold induction) of drought stress compared to day 0. After re-watering, the expression of *UO* declined (> 3-fold reduction compared to day10). In contrast, *ALN* transcript levels did not vary significantly with day or treatment over the same time frame when analyzed by one-way ANOVA. Although, it appears that *ALN* transcript decreases under drought stress (day 5 and 10) compared to the control, these are not statistically significant.



**Figure 3.8. Allantoin concentration in Col-0 leaves in response to drought and well-watered conditions.** Allantoin content in Col-0 leaves after 5 and 10 days of withholding water and on day 14 which represents 48 h after re-watering the drought-stressed plants (white bars) and in well-watered control condition (black bars). Day 0 indicates the day before withholding water. DW, dry weight. Error bars are the standard error of the mean (SEM). (n= 3).



**Figure 3.9. RT-PCR analysis of a number of ureide metabolic genes under drought stress.** Expression analysis of ureide metabolism genes in leaf tissue of Col-0 plants on day 0, 5 and 10 days after withholding water as well as two days after re-watering drought-stressed plants (day 14). *RD29A*, *RD29B* and *RD26* represent stress-responsive genes and *CSD1*, *CAT2* and *APX1* represent antioxidant enzyme genes. *ACT7* served as reference gene. *XDH*, xanthine dehydrogenase; *UO*, uricase; *ALNS*, allantoin synthase; *ALN*, allantoinase; *AAH*, allantoate amidohydrolase; *RD26/RD29A/RD19B*, dehydration-responsive genes; *CSD*, superoxide dismutase (Cu/Zn SOD); *CAT*, catalase; *APX*, ascorbate peroxidase; *ACT7*, Actin7. Image is representative of three independent replicates.



**Figure 3.10. qRT-PCR analysis of *UO* and *ALN* transcripts in Col-0 leaves under drought and well-watered conditions.** Plant leaves were sampled before withholding water (day 0) and after withholding water for 5 and 10 days. Day 14 under drought represents two days after re-watering the stressed plants. Leaves were also sampled under well-watered (control) condition for 5, 10 and 14 days. Data are representative of relative fold change of expression after normalization using *UBQ10*. Data are presented as the mean of three independent biological replicates  $\pm$  SEM. (n= 3). *UO*, uricase; *ALN*, allantoinase.

### 3.2.3. Soil gravimetric water content

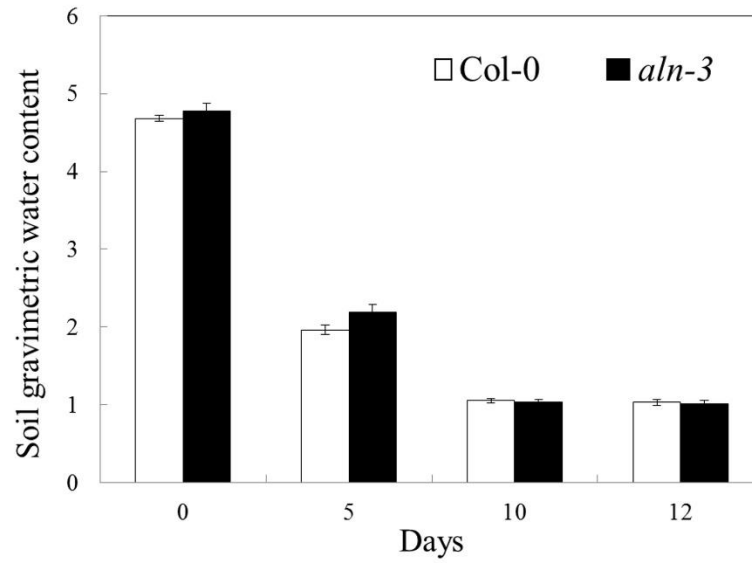
The soil gravimetric water content (SGWC) was used as a parameter to evaluate the water content of the soil corresponding to the different sampling times after withholding water (Figure 3.11). The reduction in the soil gravimetric water content did not differ significantly between the Col-0 and *aln-3* pots. The reduction in soil water on day 5 and 10 after withholding water is associated with accumulation of allantoin in the Col-0 plants as shown in Figure 3.8.

### 3.2.4. Drought stress tolerance of *aln-3* plants compared to wild-type

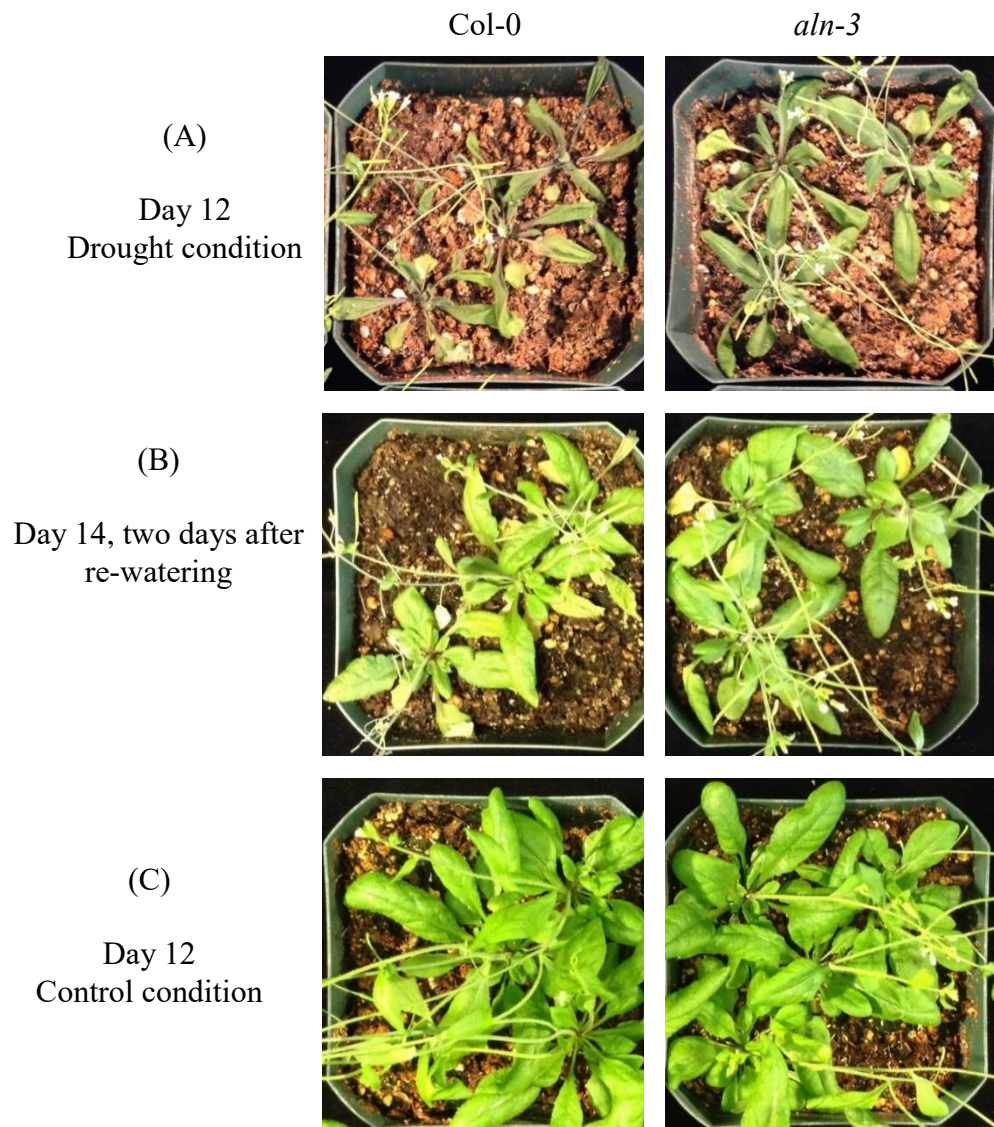
Under normal growth condition, *aln-3* did not display visual phenotypic differences in the growth or morphology compared with the Col-0. However, when 4-week-old Col-0 and *aln-3* plants were tested for resistance to drought stress, *aln-3* mutant plants were less susceptible. Higher tolerance to water stress was found for *aln-3* plants where Col-0 plants displayed more wilting symptoms (Figure 3.12). Under drought, *aln-3* plants had higher DW and FW of aerial plant part and after recovery than Col-0 plants (Figure 3.13).

Mutation in allantoinase in the *aln-3* mutant resulted in the accumulation of allantoin in the mutant. Accumulation of allantoin in *aln-3* plants was confirmed in various tissues of mature mutant plants by HPLC (Figure C8; Appendix C). No uric acid or allantoate was detected in *aln-3* plants by HPLC under control or drought conditions. Figure 3.14 shows the comparison of allantoin levels in leaves of *aln* and Col-0 under control and drought conditions. *aln-3* plants contained increased levels of allantoin ( $> 5 \mu\text{mol g DW}^{-1}$ ) prior to withholding water, which increased in response to drought. Levels of allantoin in leaf tissue of *aln-3* was much higher ( $> 12$  times on day 5 and  $> 9$  times on day 10) than Col-0 leaf tissue under drought at the same time points (Figure 3.14).

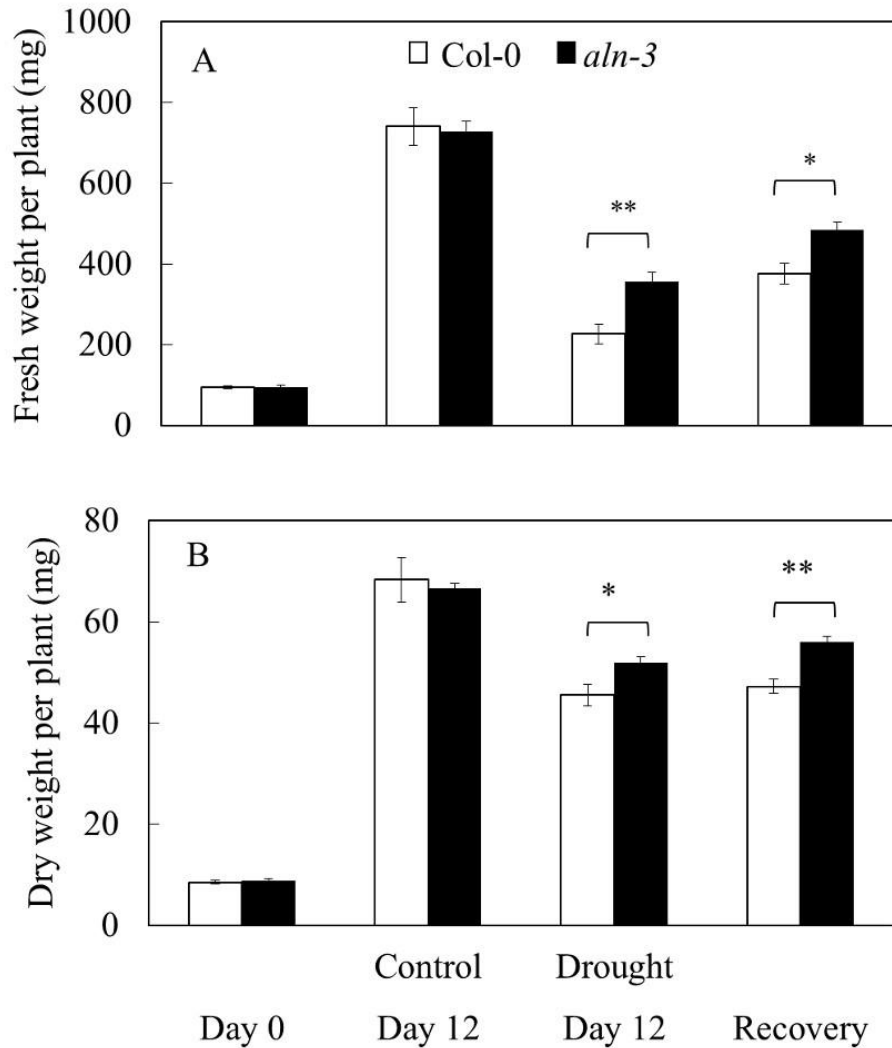




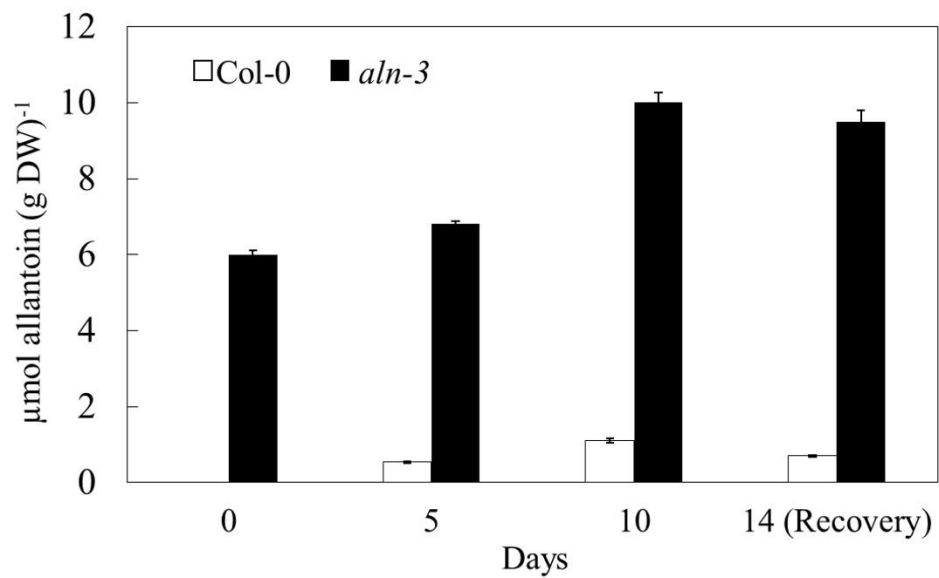
**Figure 3.11. Soil gravimetric water content (SGWC) during withholding of water.** SGWC in Col-0 (white bars) and *aln-3* plants (black bars) during withholding water. Data presented are the mean of three independent replicates  $\pm$  SEM. (n= 3)



**Figure 3.12. Phenotypes of Col-0 and *aln-3* plants following withholding of water and re-watering.** (A) Day 12 after withholding water. (B) Day 14 represents two days after re-watering the drought-stressed plants. (C) Well-watered control plants on day 12. Images are representative of three independent experiments.



**Figure 3.13. Biomass of Col-0 and *aln-3* plants following withholding and well-watered conditions.** (A) Fresh weight and (B) dry weight of the aerial part of Col-0 and *aln-3* plants were measured on day 0 and 12 days after well-watered (control) or drought stress as well as two days after re-watering of the drought-stressed plants (day 14). Data presented are the mean of three independent replicates  $\pm$  SEM. Asterisks indicate significant differences determined by a t-test (\*  $P < 0.05$ , \*\* $P < 0.01$ ). (n= 3)



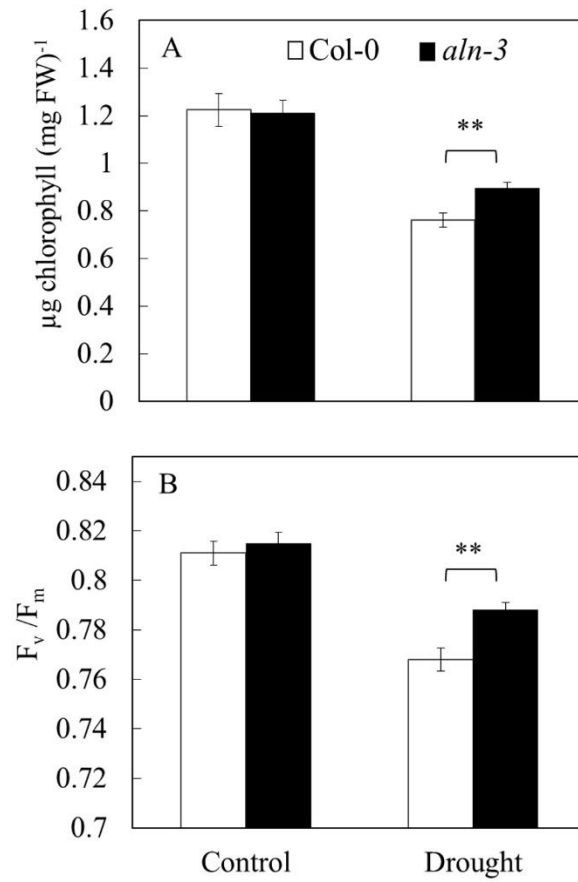
**Figure 3.14. Allantoin level in Col-0 and *aln-3* in response to drought.** Allantoin was determined in leaves of Col-0 (white bars) and *aln-3* (black bars) after 0, 5 and 10 days of withholding water and on day 14, two days after re-watering. The data for Col-0 are the same as depicted in Figure 3.8, shown here for comparison. Data presented are the mean of three independent replicates  $\pm$  SEM. (n= 3)

### 3.2.5. Chlorophyll content and the photochemical efficiency of PSII

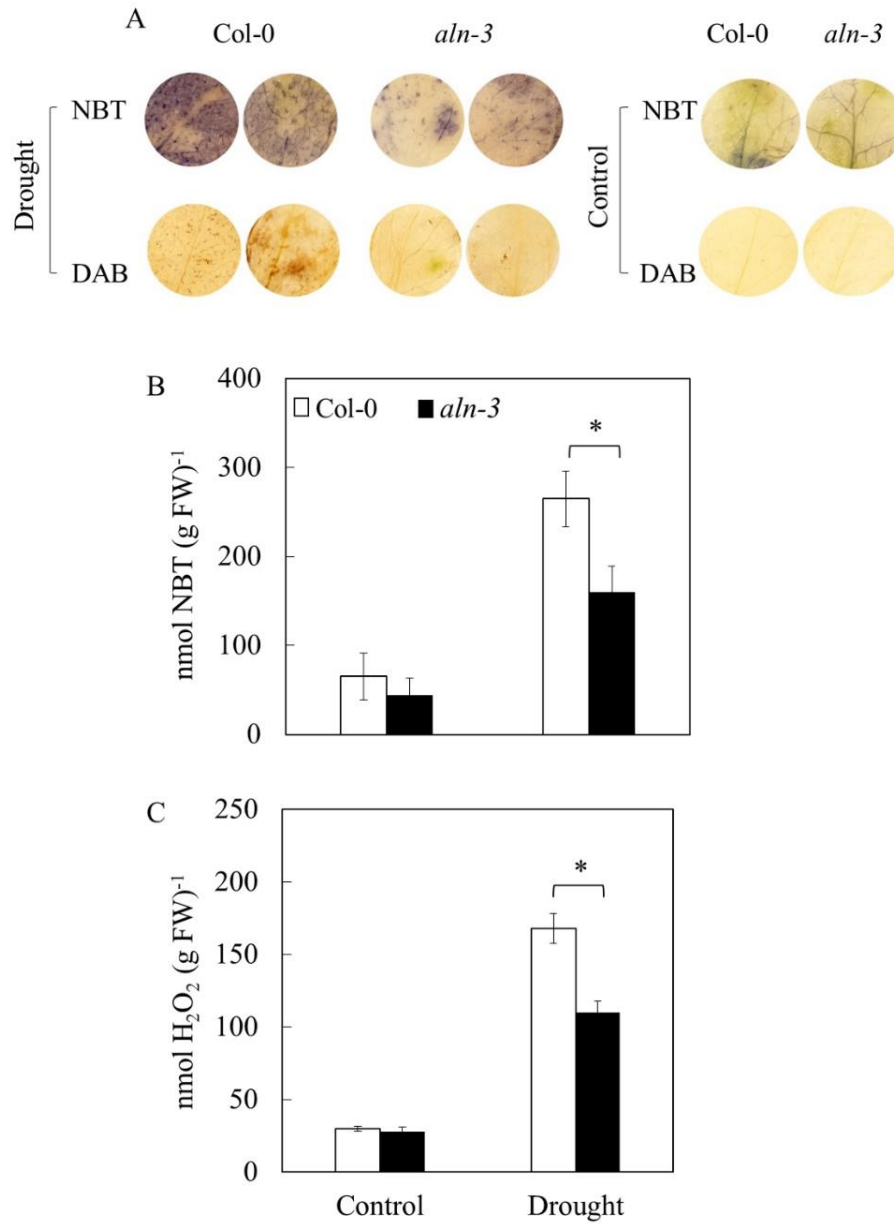
To investigate the physiological effects of elevated allantoin concentration following water stress, total chlorophyll content and photochemical efficiency of PSII ( $F_v/F_m$  ratio) were determined (Figure 3.15). Total chlorophyll levels and the  $F_v/F_m$  ratio did not significantly differ in Col-0 and *aln-3* leaves under normal growth conditions. However, both chlorophyll amount and  $F_v/F_m$  ratio showed greater reduction in water-stressed Col-0 leaves compared to *aln-3* leaves after drought treatment.

### 3.2.6. Levels of $O_2^-$ and $H_2O_2$ on day 10 of withholding water

To address the hypothesis that allantoin protects against abiotic stress, the levels of superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) in leaves of Col-0 and *aln-3* plants were determined. Histochemical detection of  $O_2^-$  and  $H_2O_2$  and quantification was performed using nitroblue tetrazolium (NBT) and diaminobenzidine (DAB), respectively. Compared with Col-0, weaker staining was observed in *aln-3* leaves for both  $O_2^-$  and  $H_2O_2$  on day 10 of drought stress (Figure 3.16 A). *aln-3* leaves contained significantly lower concentrations of both  $O_2^-$  and  $H_2O_2$  after 10 days of drought stress (Figure 3.16 B,C).



**Figure 3.15. Chlorophyll content and maximum efficiency of PSII.** (A) Chlorophyll content and (B) maximum efficiency of PSII photochemistry ( $F_v/F_m$  ratio) of Col-0 and *aln-3* leaves were measured after 10 days of control (watered) and drought stress. Data presented are the mean of three independent replicates  $\pm$  SEM. Asterisks indicate significant differences determined by a t-test (\*\* $P < 0.01$ ). (n= 5)



**Figure 3.16. Staining and quantification of  $O_2^-$  and  $H_2O_2$ .** (A) Visualization of  $O_2^-$  and  $H_2O_2$  detected by NBT and DAB histochemical staining, respectively. Col-0 and *aln-3* leaves were stained after 10 days of drought stress and compared to controls. (B) Quantification of  $O_2^-$  and (C)  $H_2O_2$  in leaf tissue under the same conditions as (A). Levels of  $O_2^-$  is expressed as nmol of reduced NBT (g FW)<sup>-1</sup>. Data presented are the mean of three independent replicates  $\pm$  SEM. Asterisks indicate significant differences determined by a t-test (\*  $P < 0.05$ ). (n = 3)

### 3.3. Increased light intensity

#### 3.3.1. Ureide concentration in wild-type leaves after light treatments

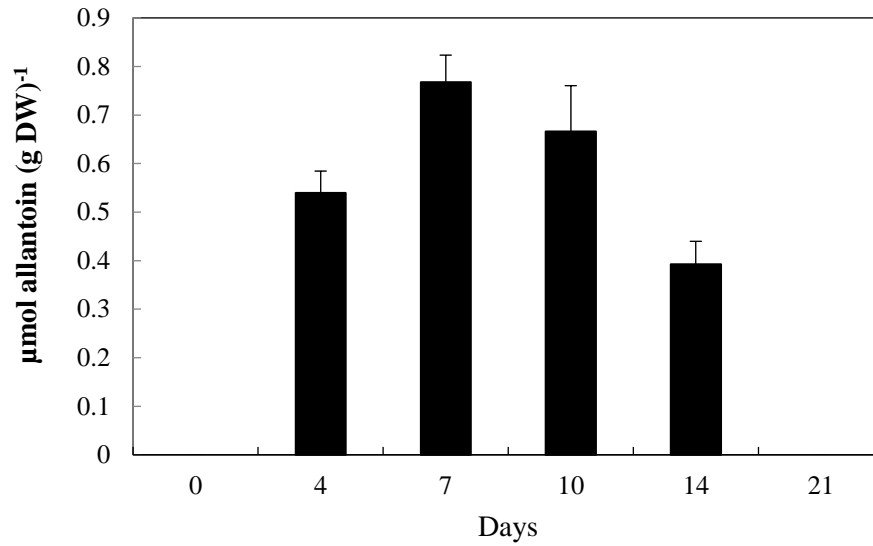
In order to quantify ureide concentration in wild-type *Arabidopsis* leaves under moderate and increased light treatments, Col-0 seeds were allowed to germinate on MS media for 7 days and the seedlings transferred to pots with soil to grow under standard *Arabidopsis* growth condition ( $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; day/night temperature  $22^{\circ}\text{C}$  and a light/dark cycle of 16/8 h) for an additional 10 days. The 17-day-old plants were shifted to moderate or increased light conditions. The ureide content of leaf tissue of Col-0 plants under light treatments was determined using HPLC.

No uric acid and allantoate were detected in leaf tissue of Col-0 plants in response to light treatments. However, increased allantoin ( $0.496 \mu\text{mol (g DW)}^{-1}$ ) was found in the leaves on day 4 after transferring plants to the increased light condition (Figure 3.17). Allantoin levels reached a maximum level of  $0.72 \mu\text{mol (g DW)}^{-1}$  on day 7, decreasing to 0.578 and  $0.346 \mu\text{mol (g DW)}^{-1}$  on days 10 and 14 respectively. Three weeks after shifting the plants to increased light condition, no allantoin was detected in the leaves (Figure 3.17). However, plant leaf tissue was usually completely purple and whole-plant senescence had occurred by day 21 of increased light treatment. There was no detectable allantoin or other ureides in leaf samples that were taken at the same time points from plants under moderate light condition.

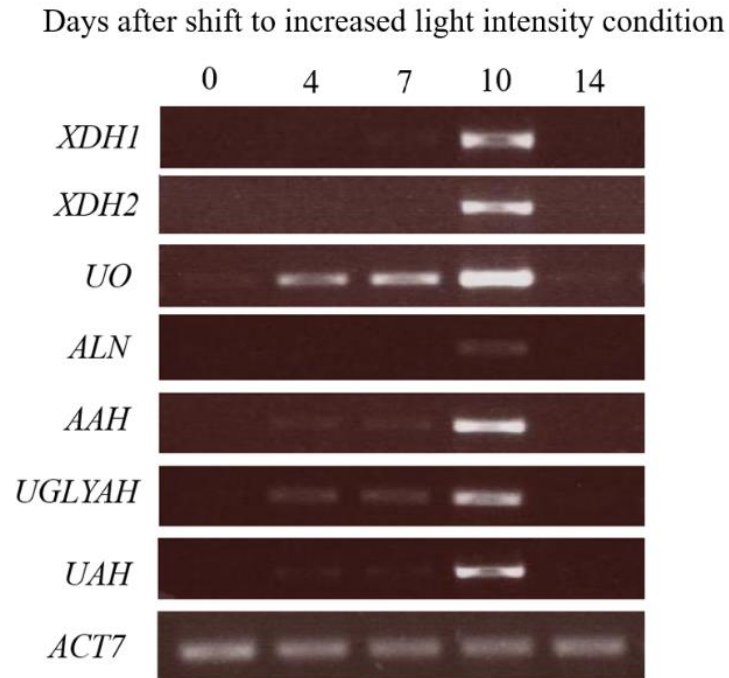
#### 3.3.2. Expression analysis of ureide genes by RT-PCR

Expression of seven genes (*XDH1*, *XDH2*, *UO*, *ALN*, *AAH*, *UGLYAH* and *UAH*) involved in ureide metabolism was analyzed by RT-PCR to determine whether expression was influenced by light treatment. In response to increased light intensity, the relative expression of *UO* started to increase on day 4 and continued up to day 10. The expression of all other ureide genes also increased by day 10. However, expression of *ALN* showed less induction compared to the other ureide metabolism genes. By day 14 of increased light intensity, expression of all genes was reduced (Figure 3.18). When expression of ureide genes was analyzed by RT-PCR after 10 days of moderate- and increased- light treatments, the samples under increased light generally showed higher expression. (Figure C9; Appendix C).





**Figure 3.17. Allantoin accumulation in Col-0 leaves in response to increased light intensity.** Allantoin content detected by HPLC in Col-0 leaves after 0, 4, 7, 10, 14 and 21 days of increased light intensity treatment. Plants were grown on standard Arabidopsis growth condition for 17 days before increased light treatment. Day 0 indicates the day before increased light treatment. DW, dry weight. Error bars are the standard error of the mean (SEM). (n= 3)



**Figure 3.18. RT-PCR analysis of ureide metabolism genes under increased light condition.** Expression analysis of ureide metabolism genes in leaf tissue of Col-0 plants on day 0, 4, 7, 10 and 14 after transferring the 17-day-old plants to a chamber with increased light condition. *ACT7* served as reference gene. *XDH*, xanthine dehydrogenase; *UO*, uricase; *ALN*, allantoinase; *AAH*, allantoate amidohydrolase; *UGLYAH*, ureidoglycine aminohydrolase; *UAH*, ureidoglycolate amidohydrolase; *ACT7*, Actin7. Image is representative of three independent experiments.

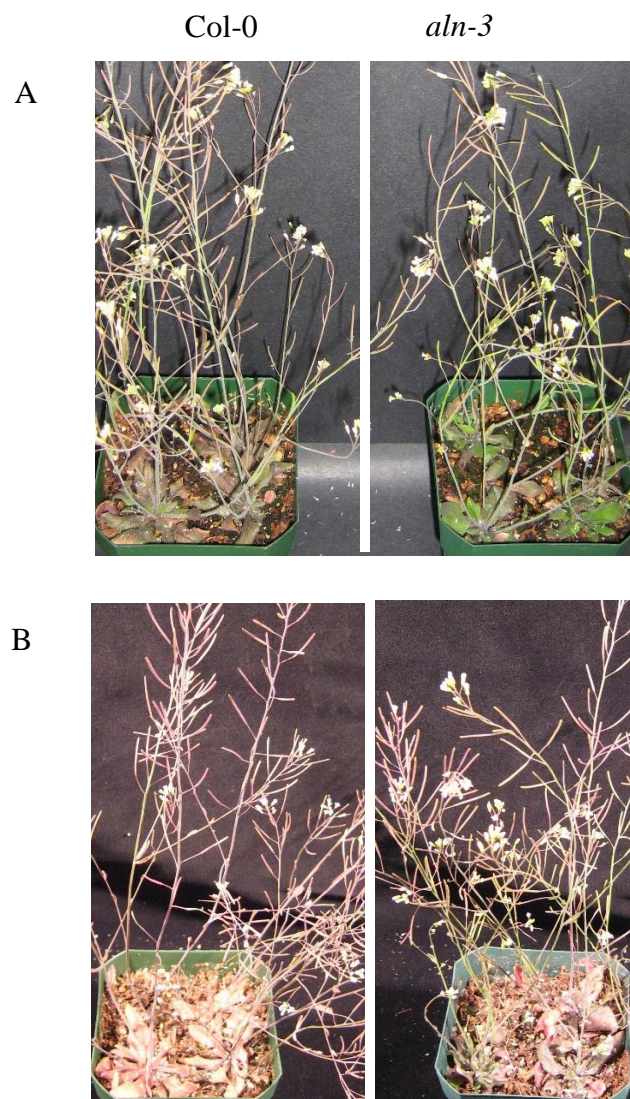
### 3.3.3. Increased light stress tolerance of *aln-3* plants compared to wild-type

After seven days of increased light intensity, plants started to show phenotypical changes compared to plants in moderate light. The main visible morphological changes under increased light condition included thickened leaves, reduced chlorophyll and enhanced levels of anthocyanin which resulted in purple leaf colour. On day 14 after increased light treatment, Col-0 plants lost more chlorophyll and accumulated more purple pigments (anthocyanin) compared to *aln-3* plants (Figure 3.19A). On the third week of the increased light treatment, most of the plants were completely purple and dried (Figure 3.19B). Figure 3.20 shows the phenotypes of plants on day 14 (A) and 21 (B) of moderate light treatment. No visual difference in the phenotype of Col-0 and *aln-3* was found under moderate light condition.

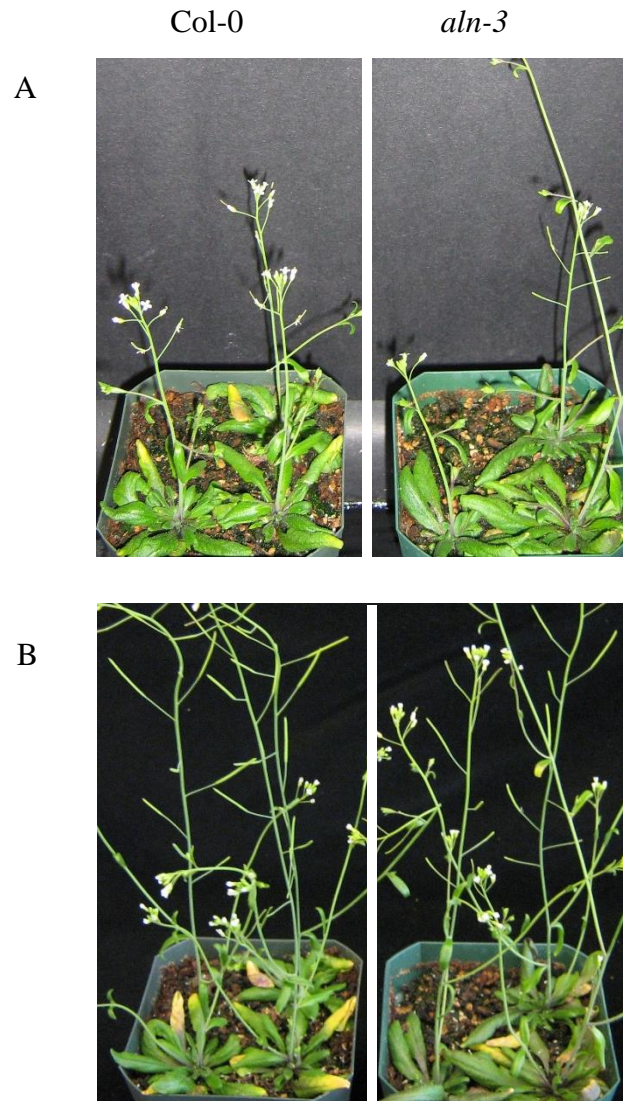
Ureide concentration was also measured in *aln-3* mutant plants under increased light intensity condition. Only allantoin and uric acid were detected in *aln-3* leaves under increased light. Allantoin content in the mutant was increased incrementally to a maximum amount on day 21. Uric acid content showed an increase until day 10 but it was not detectable on day 21 (Figure 3.21).

### 3.3.4. Chlorophyll and anthocyanin content and the maximum efficiency of PSII

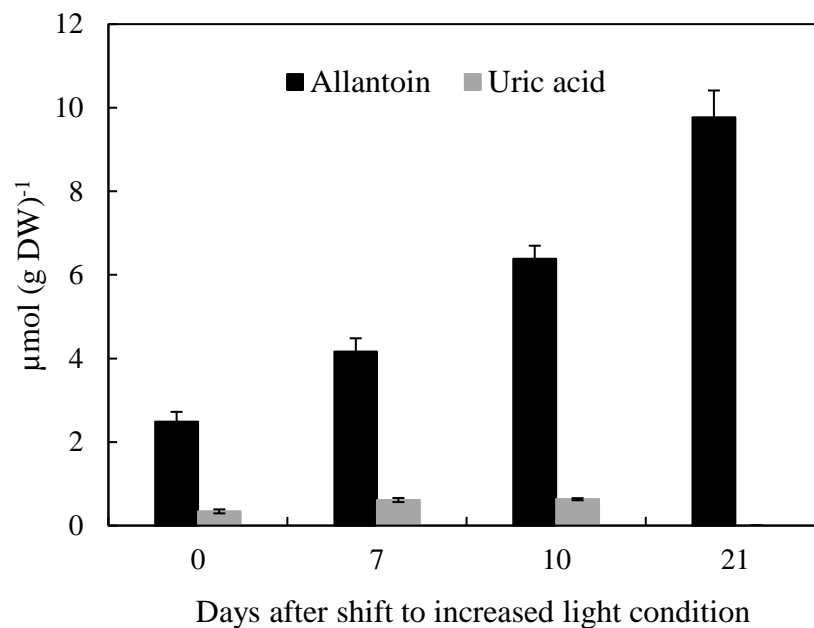
The level of total chlorophyll and anthocyanin as well as the maximum efficiency of PSII ( $F_v/F_m$  ratio) were detected in the leaves of Col-0 and *aln-3* plants after 0, 7, 14 and 21 days treatment under moderate and increased light conditions. Day 0 represents the day before starting the moderate or increased light treatments. None of the chlorophyll,  $F_v/F_m$  ratio or anthocyanin values were significantly different on tested time points for the Col-0 and *aln-3* plants under moderate light intensity when tested by student t-test. However, the level of total chlorophyll and  $F_v/F_m$  ratio were significantly higher in *aln-3* on day 14 of increased light treatment compared to Col-0 (Figure 3.22 A, B). On the other hand, the anthocyanin level was significantly higher in Col-0 leaves compared to *aln-3* on day 14 after increased light treatment (Figure 3.22 C). The raw data for each measurement under moderate and increased light are available in Table B7, Appendix B.



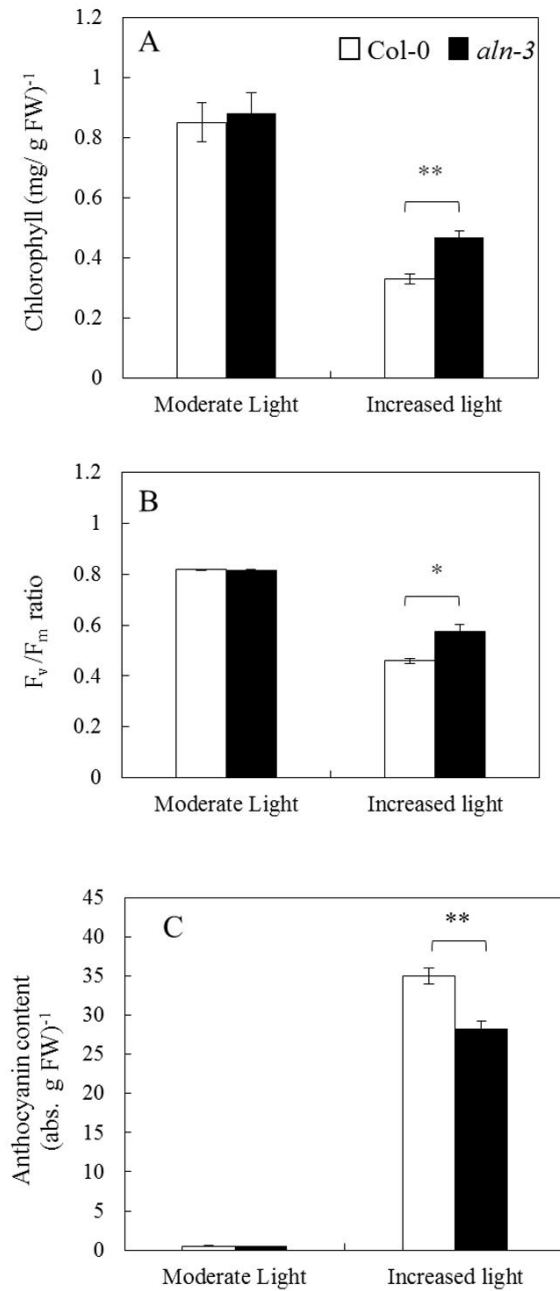
**Figure 3.19. The effect of increased light condition on the phenotype of Col-0 and *aln-3* plants.** Plants were grown under *Arabidopsis* standard growth conditions for 17 days. Then, 17-day-old plants were shifted to a chamber with increased light intensity condition. Representative images show the plants on day 14 (A) and 21 (B) after shifting to the chamber with increased light condition.



**Figure 3.20. The effect of moderate light condition on the phenotype of Col-0 and *aln-3* plants.** Plants were grown for 17 days under Arabidopsis standard growth conditions before shifting to moderate light intensity condition. Representative images show plants on day 14 (A) and 21 (B) after shifting to the moderate light condition.



**Figure 3.21. Allantoin and uric acid content in *aln-3* leaves under increased light conditions.** Concentration of allantoin and uric acid in leaves of *aln-3* on day 0, 7, 10 and 21 after shifting 17-day-old plants to increased light conditions. Day 0 represents the day before shifting the plants to the increased light condition. Data presented are the mean of three independent replicates  $\pm$  SEM. (n= 3)



**Figure 3.22. Levels of chlorophyll and anthocyanin and the maximum efficiency of PSII ( $F_v/F_m$  ratio) on day 14 after moderate and increased light treatments.** 17-day-old Col-0 (white bars) and *aln-3* (black bars) plants were shifted to moderate or increased light conditions. The data show the chlorophyll content (A),  $F_v/F_m$  ratio (B), and anthocyanin content (C) in Col-0 and *aln-3* leaves, 14 days after light treatments. Data presented are the mean of three independent replicates  $\pm$  SEM. Asterisks indicate significant differences determined by a t-test (\*  $P < 0.05$ , \*\* $P < 0.01$ ). (n= 3). abs; absorption.

### 3.4. NaCl and mannitol treatments

#### 3.4.1. Ureide concentration in wild-type seedlings

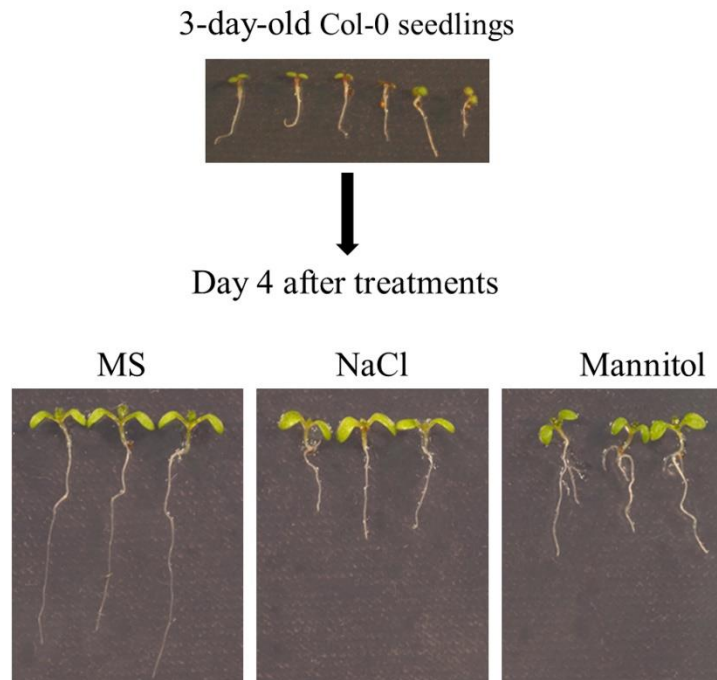
The concentration of uric acid, allantoin, and allantoate in Col-0 seedlings exposed to NaCl or osmotic stress were measured using HPLC. Col-0 seeds were first germinated on MS media and 3-day-old seedlings were transplanted to plates containing 200 mM NaCl or 250 mM mannitol. Seedlings were also moved to NaCl- and mannitol-free MS plates as a control (Figure 3.23). Transfer to either NaCl or mannitol resulted in phenotypic changes including, reduced root elongation and smaller leaves in the stress treated seedlings (Figure 3.23).

There were no detectable ureides in 3-day-old seedlings before transplantation. When the levels of ureides in seedlings was analyzed four days after treatments, both allantoin and allantoate were detected (Figure 3.24). Allantoin content increased by 5- and 5.4-fold in seedlings four days after NaCl and mannitol treatments, respectively. Allantoate levels also increased in response to both treatments, with a significant increase in response to mannitol (Figure 3.24). Ureide content was also determined every two hours after moving the seedlings to NaCl or mannitol plates for the first 10 hours after transfer, but no significant amount of ureides were found by HPLC.

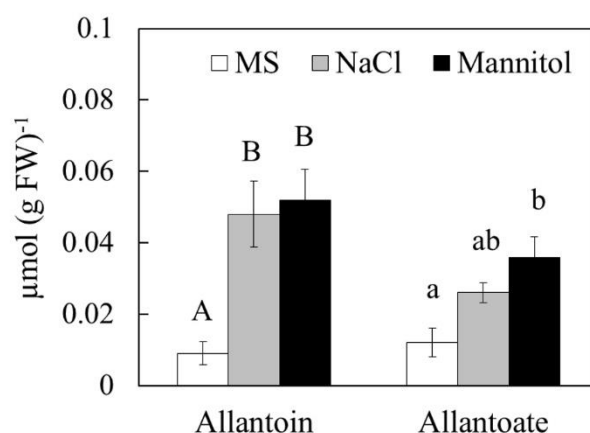
#### 3.4.2. Expression analysis of ureide genes in wild-type seedlings

When higher concentrations of allantoin and allantoate were observed in Col-0 seedlings four days after NaCl and mannitol treatments compared to control, the expression of ureide metabolism genes were also analyzed by RT-PCR. The expression of these genes was analyzed four days after shifting the 3-day-old Col-0 seedlings to the NaCl, mannitol, and control (MS) media. Lower expression of *ALN* was observed in Col-0 seedlings after treatment with NaCl or mannitol. *AAH* expression was repressed by NaCl and *ALNS* was also slightly reduced in NaCl treated seedlings. Both *XDHI* and *UO* were unaffected by the treatments (Figure 3.25). Higher expression of *RD26* was observed in seedlings under both stress conditions compared to control. *RD26* was used as a positive stress-inducible gene since significant increase in the expression of this gene has been reported in *Arabidopsis* following NaCl (Fujita *et al.*, 2004) and mannitol (Fujii *et al.*, 2011) treatments.

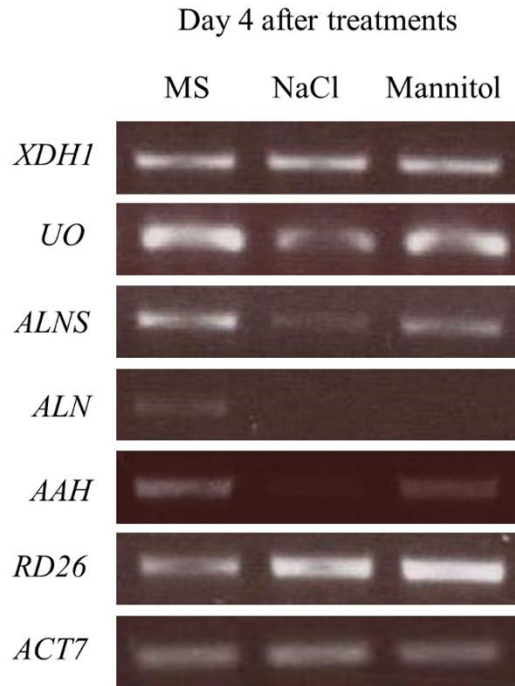




**Figure 3.23. Phenotype of Col-0 seedlings before and 4 days after NaCl and mannitol treatments.** Representative image of 3-day-old Col-0 seedlings before and after transfer to MS media containing 200 mM NaCl or 250 mM mannitol for 4 days. The same media without NaCl and mannitol was used as control (MS).



**Figure 3.24. Ureide concentration in Col-0 seedlings response to NaCl and mannitol treatments.** Ureides (allantoin and allantoate) level in Col-0 seedlings in response to 200 mM NaCl and 250 mM mannitol treatments for four days as well as control MS treatment. Data presented are the mean of three independent replicates  $\pm$  SEM. Different letters denote significant differences between treatments determined by Tukey HSD test. (n= 3).



**Figure 3.25. RT-PCR analysis of ureide genes, four days after NaCl and mannitol treatments.** Representative image shows the gene expression analysis of a number of ureide genes in Col-0 seedlings in response to 200 mM NaCl, 250 mM mannitol and control (MS) treatments. 3-day-old Col-0 seedlings were shifted to these media and were allowed to grow for 4 days before sampling. For stress-responsive marker gene and reference gene, *RD26* and *ACTIN7* were served, respectively. *XDH*, xanthine dehydrogenase; *UO*, uricase; *ALNS*, allantoin synthase; *ALN*, allantoinase; *AAH*, allantoate amidohydrolase; *RD26*, dehydration-responsive; *ACT7*, Actin7. (n= 3)

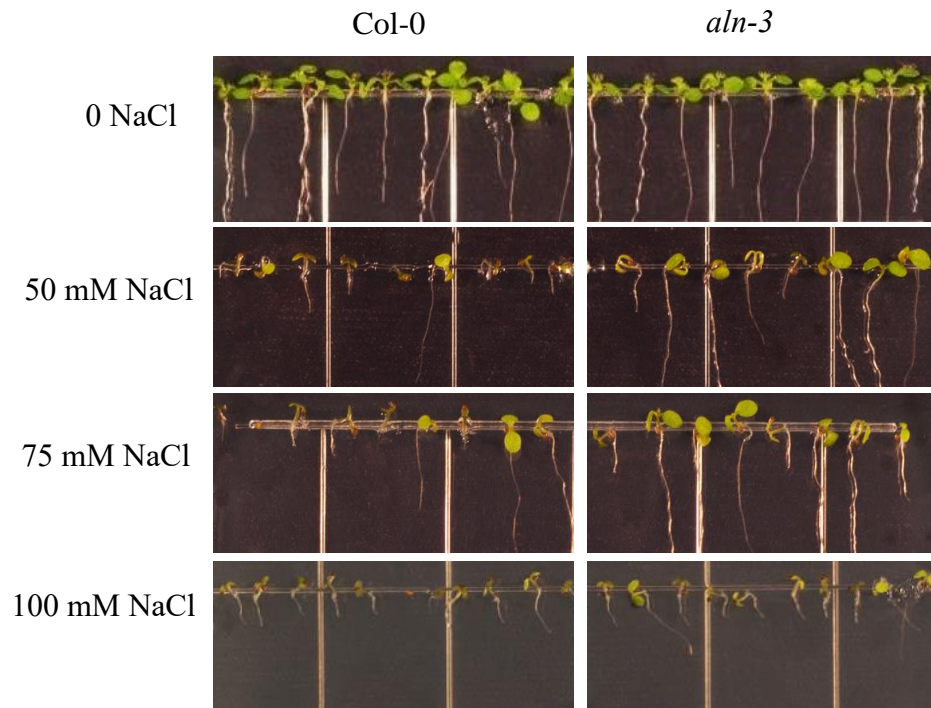
### **3.5. NaCl tolerance of *aln-3* seedlings compared to wild-type**

#### **3.5.1. Col-0 and *aln-3* seedling tolerance when germinated on NaCl**

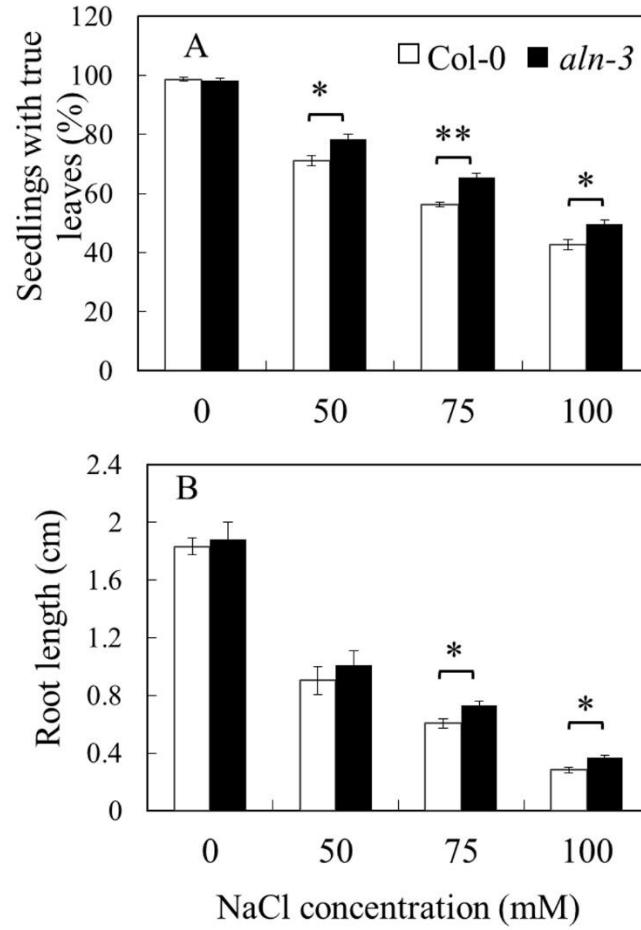
Col-0 and *aln-3* seeds were plated on MS media containing NaCl concentrations of 50 mM, 75 mM and 100 mM as well as a 0 mM NaCl control. The percentage of germinated seeds did not differ between Col-0 and *aln-3* under any of the tested media (Figure C10; Appendix C). However, Col-0 seedlings had slower shoot and primary root growth in NaCl when visually compared to *aln-3* seedlings (Figure 3.26). Therefore, the root length and the percentage of seedlings with true leaves were measured under all of the tested media on day 7 after germination of seeds (Figure 3.27). A greater number of *aln-3* seedlings showed true leaf development (Figure 3.27 A), as well as longer root length (Figure 3.27 B) than the Col-0 under 50, 75 and 100 mM NaCl concentrations.

#### **3.5.2. Seedlings stress tolerance when transferred to NaCl media**

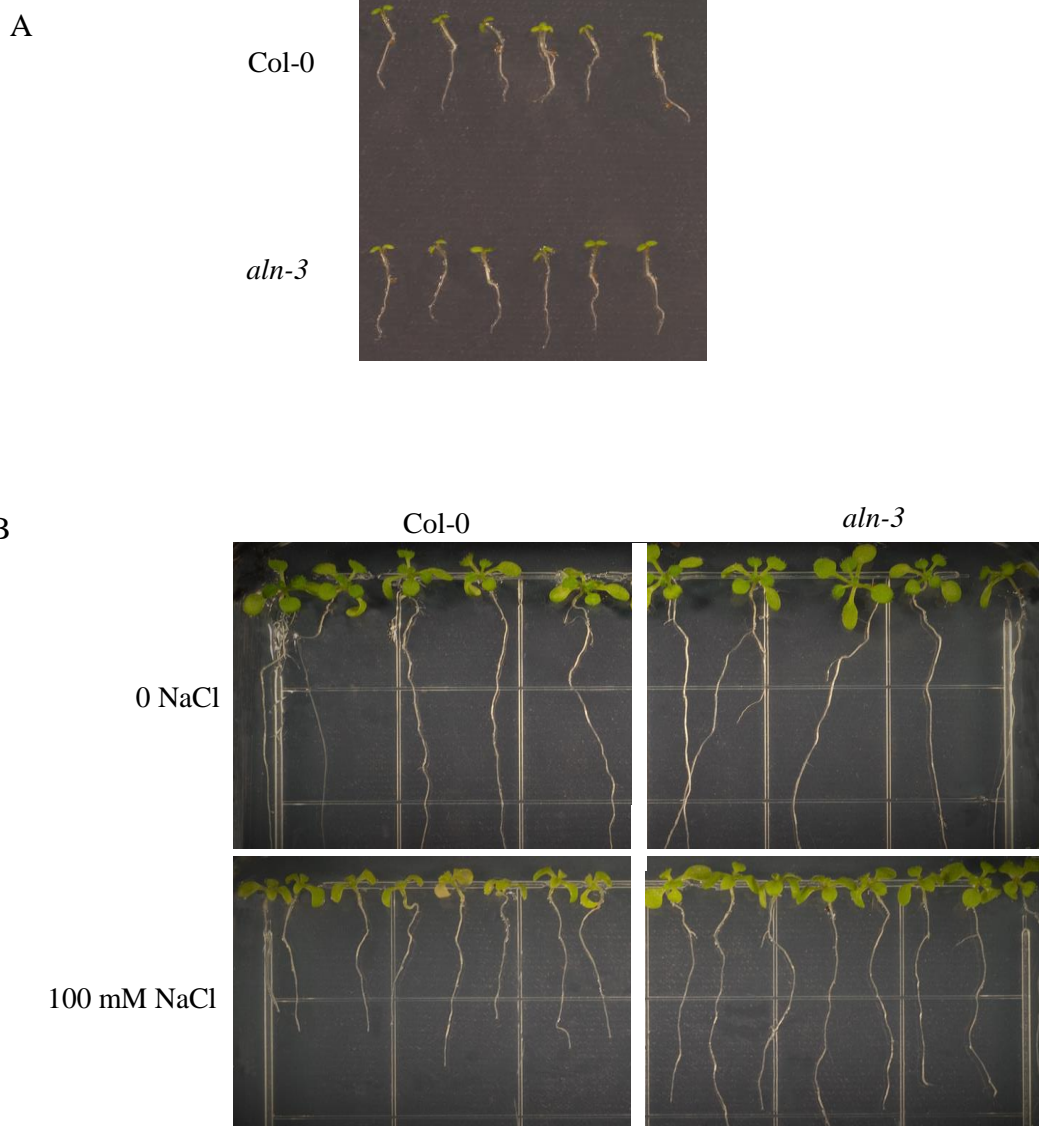
To assess the NaCl stress tolerance of seedlings, Col-0 and *aln-3* seeds were first germinated on MS media for five days and then were transferred to new plates containing MS media without NaCl and with 100 mM NaCl. Seedlings of Col-0 and *aln-3* had the same root length and weight when transferred to the new media (Figure 3.28A). Seedlings were allowed to grow for an additional nine days and then their growth was assessed. The fresh weight and primary root length of seedlings were measured since they were visually different in the 2-week-old Col-0 and *aln-3* seedlings grown on 100 mM NaCl (Figure 3.28B). *aln-3* seedlings exhibited significantly higher fresh weight and root length than Col-0 seedlings under 100 mM NaCl (Figure 3.29).



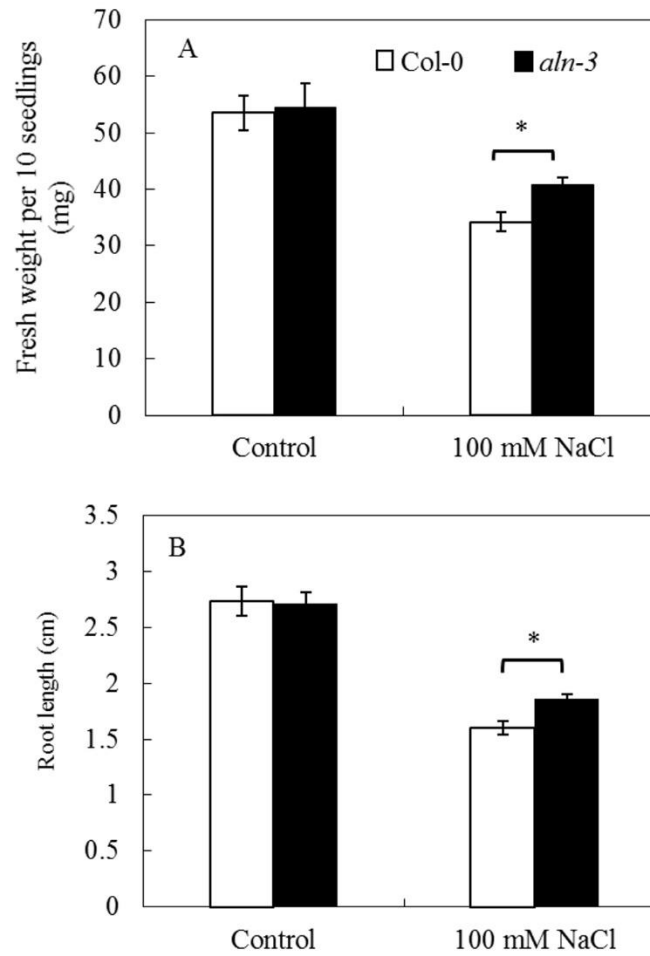
**Figure 3.26. Phenotypes of Col-0 and *aln-3* seedlings following germination under NaCl stress.** Col-0 and *aln-3* seeds were planted on MS media containing 50, 75 and 100 mM of NaCl and the control media without NaCl. The image shows seedling phenotype seven days after germination. Image is representative of three independent experiments.



**Figure 3.27. Percentage of seedlings with true leaves and the root length of seedlings germinated on NaCl media for 7 days.** (A) Percentage of Col-0 and *aln-3* seedlings with developed true leaves on day 7 after germination under MS media with 0, 50, 75 and 100 mM NaCl. (B) Primary root length of Col-0 and *aln-3* seedlings on day 7 after germination on MS media with 50, 75 and 100 mM and without NaCl. Measurements were made from at least 20 seedlings per replicate. Data presented are the mean of three independent replicates  $\pm$  SEM. Asterisks indicate significant differences determined by a t-test (\*  $P < 0.05$ , \*\* $P < 0.01$ ). (n= 3)



**Figure 3.28. Phenotype of seedlings before and after shift to the media with 100 mM NaCl and without NaCl** (A) Phenotypes of 5-day-old Col-0 and *aln-3* seedlings before transferring to the new media. (B) 5-day-old seedlings were placed on MS media with and without 100 mM NaCl and were allowed to grow for nine more days. The figure shows a representative phenotype of 2-week-old Col-0 and *aln-3* seedlings grown on media containing 100 mM NaCl and 0 NaCl as control. Images are representative of three independent experiments.



**Figure 3.29. Fresh weight and root length of 2-week-old seedlings under 0 and 100 mM NaCl.** 5-day-old Col-0 and *aln-3* seedlings were trans-placed in MS media with and without 100 mM NaCl and were allowed to grow for nine additional days. (A) Fresh weight of Col-0 and *aln-3* seedlings was measured after nine days under 0 and 100 mM NaCl. Data represent the fresh weight (as mg) per 10 seedlings. (B) Primary root length of 2-week-old Col-0 and *aln-3* seedlings, nine days after trans-placing the seedlings to media with 0 and 100 mM NaCl. Measurements were made of at least 30 seedlings per experiment. Data presented are the mean of four independent replicates  $\pm$  SEM. Asterisks indicate significant differences determined by a t-test (\*  $P < 0.05$ ). (n= 4)

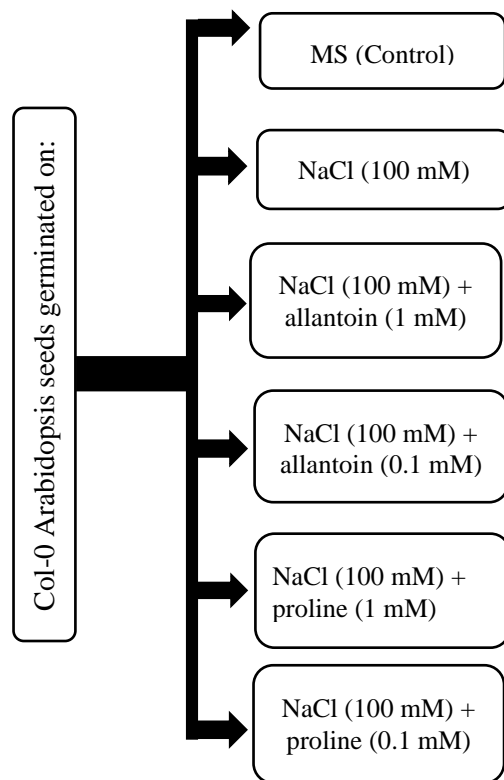


### 3.6. Effect of exogenous allantoin on the tolerance of wild-type seedlings under NaCl stress

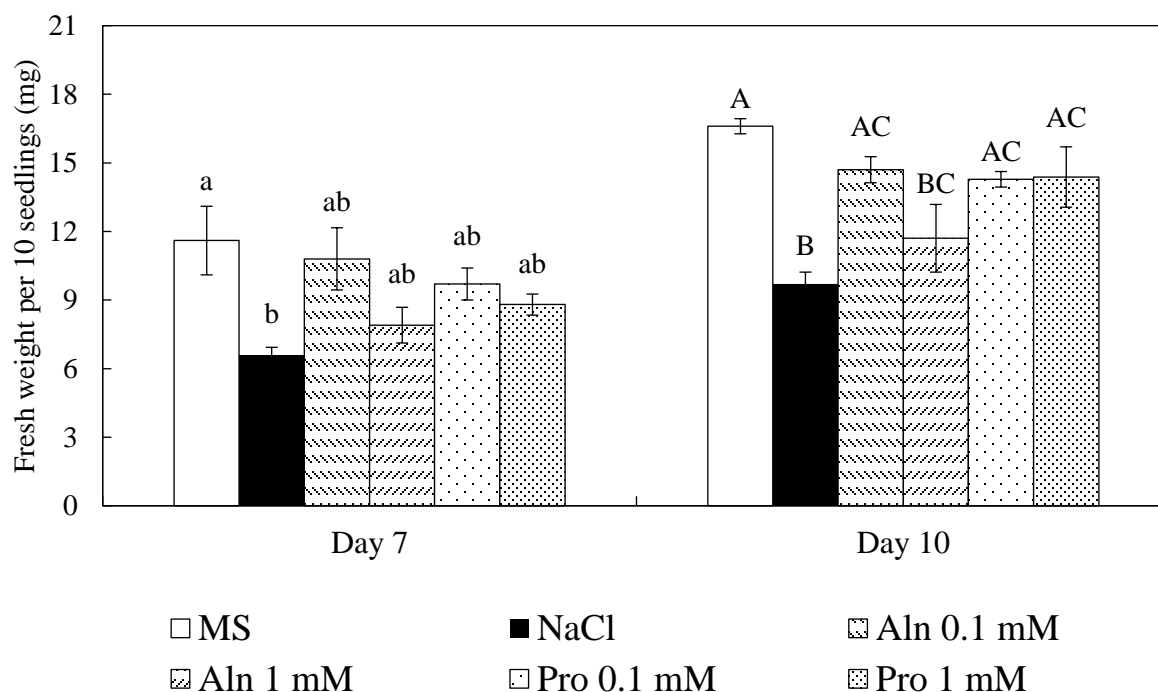
#### 3.6.1. Effect of exogenous allantoin when seeds are germinated on NaCl

The effect of exogenous allantoin application on stress tolerance of wild-type *Arabidopsis* was tested under 100 mM NaCl. For the first set of experiments, Col-0 seeds were germinated on 100 mM NaCl and control MS media without NaCl. Two concentrations of allantoin (0.1 mM and 1 mM) were added to NaCl plates to assess the effect of exogenous allantoin on seedling NaCl stress tolerance. These concentrations were chosen based on the exogenous concentrations of allantoin that were used by Watanabe *et al.*, (2014a) and the effect of both concentrations on up-regulation of some stress genes (e.g.; dehydration-responsive genes) and ABA biosynthesis genes (Watanabe *et al.*, 2014a). Proline, which is one of the best known osmolytes in plants (Ashraf and Foolad, 2007), was used in this study also at concentrations of 0.1 mM and 1 mM, to provide a control. The basic experimental design and various tested media are shown in Figure 3.30.

When Col-0 seeds were germinated, the percentage and rate of germination was not considerably different between the various NaCl + additive media but significantly different to MS (Figure C10; Appendix C). Fresh weight of seedlings from each treatment was measured on day 7 and day 10 after germination (Figure 3.31). On day 10, there was no difference between allantoin and proline treatments. However, the seedlings in NaCl media + 1 mM allantoin were not significantly different from the NaCl treatment alone. In addition, the seedlings in NaCl + 0.1 mM allantoin and NaCl + 0.1 and 1 mM of proline did not show significant fresh weight difference with the seedlings on control MS media (Figure 3.31). The primary root length of seedlings was measured on day 10. Statistical analysis also showed that on 1 mM proline, the positive effect on seedlings' root length was comparable to the effect of 0.1 mM allantoin (Figure 3.32). The root length of seedlings in these two media was comparable to seedlings grown on the MS control without NaCl (Figure 3.32). Figure 3.33 shows the phenotype of seedlings that were germinated on NaCl, NaCl + 0.1 mM allantoin and NaCl + 1 mM proline media for 10 days. As shown in the figure, the seedlings on 100 mM NaCl have smaller roots and less developed leaves compared to the ones on 100 mM NaCl + 0.1 mM allantoin or + 1 mM proline media.

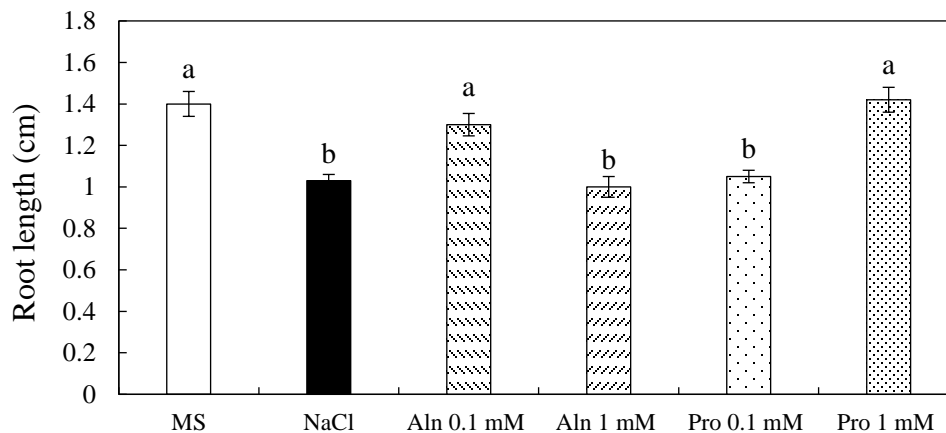


**Figure 3.30. Schematic experimental design to detect the effect of exogenous allantoin on Col-0 when germinated on NaCl.** The Col-0 seeds were planted on six different media including: Control without NaCl (MS), 100 mM NaCl, 100 mM NaCl + 0.1 mM allantoin, 100 mM NaCl + 1 mM allantoin, 100 mM NaCl + 0.1 mM proline and 100 mM NaCl + 1 mM proline.

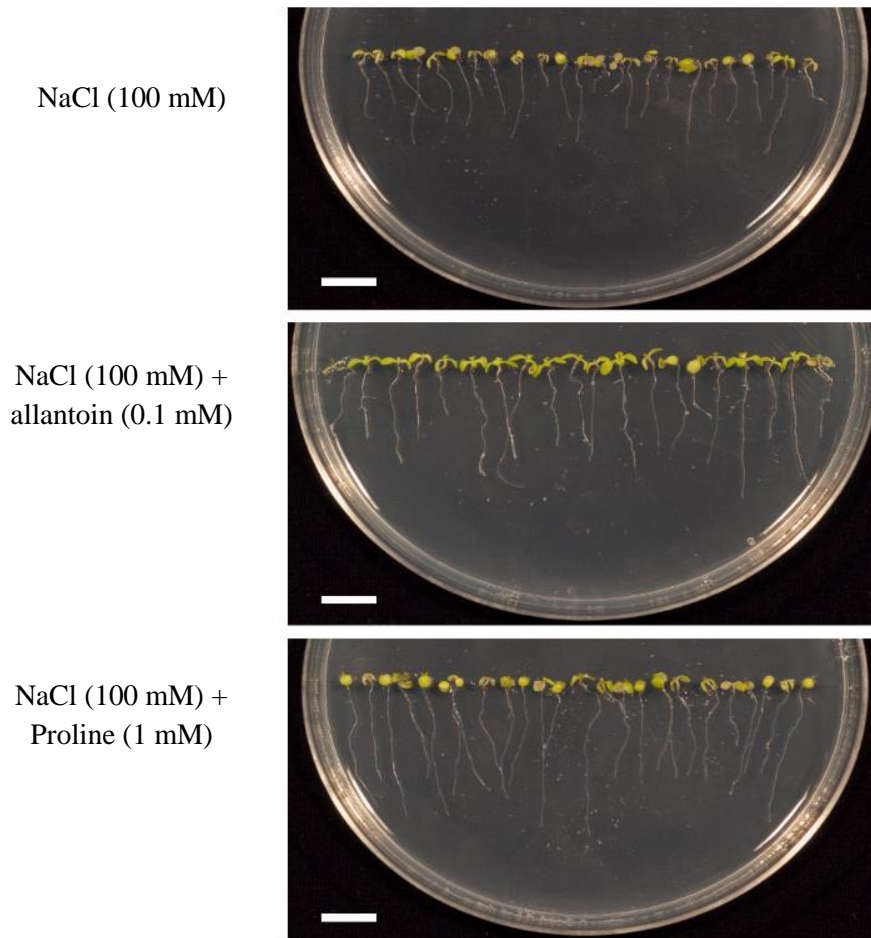


**Figure 3.31. Fresh weight of Col-0 seedlings after 7 and 10 days of germination on six different media.**

Data represent the fresh weight as mg per 10 seedlings. MS, media without NaCl; NaCl media containing 100 mM NaCl. Plates containing 0.1 or 1 mM allantoin or 0.1 or 1 mM proline also contained 100 mM NaCl. Measurements were made of at least 30 seedlings per experiment. Data presented are the mean of three independent replicates  $\pm$  SEM. Data was first analyzed by one-way analysis of variance (ANOVA) at ( $p < 0.05$ ), followed by Tukey's test to compare the means of each treatment to the means of other treatments. Different letters above the bars indicate significant differences detected by Tukey HSD test at  $p < 0.05$ . (n= 3). Aln: allantoin; Pro: proline.



**Figure 3.32. Root length of Col-0 seedlings, 10 days after germination on six different media.** MS, media without NaCl; NaCl media containing 100 mM NaCl. Plates containing 0.1 or 1 mM allantoin or 0.1 or 1 mM proline also contained 100 mM NaCl. Measurements were made of at least 20 seedlings per experiment. Data presented are the mean of three independent replicates  $\pm$  SEM. Data was analyzed by one-way analysis of variance (ANOVA) at ( $p < 0.05$ ), followed by Tukey's test to compare the means of every treatment to the mean of every other treatment. Different letters indicate statistical significant difference detected by Tukey HSD test at  $p < 0.05$ . ( $n = 3$ ). Aln: Allantoin; Pro: Proline.

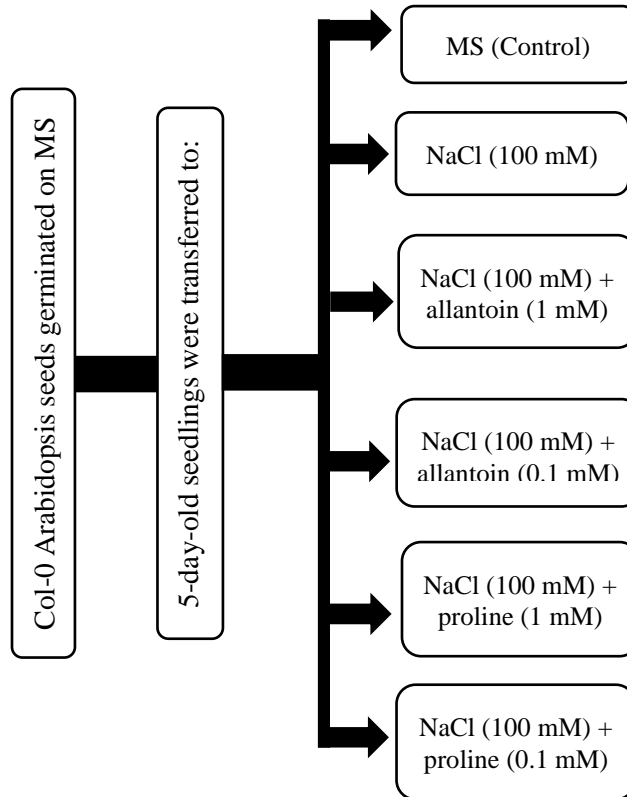


**Figure 3.33. Phenotype of 10-day-old Col-0 seedlings germinated under three supplemental NaCl media.** Col-0 seedlings were germinated for 10 days under 100 mM NaCl, 100 NaCl with 0.1 mM allantoin or 100 NaCl with 1 mM proline. The image is representative of three independent experiments. Scale bar, 1 cm.

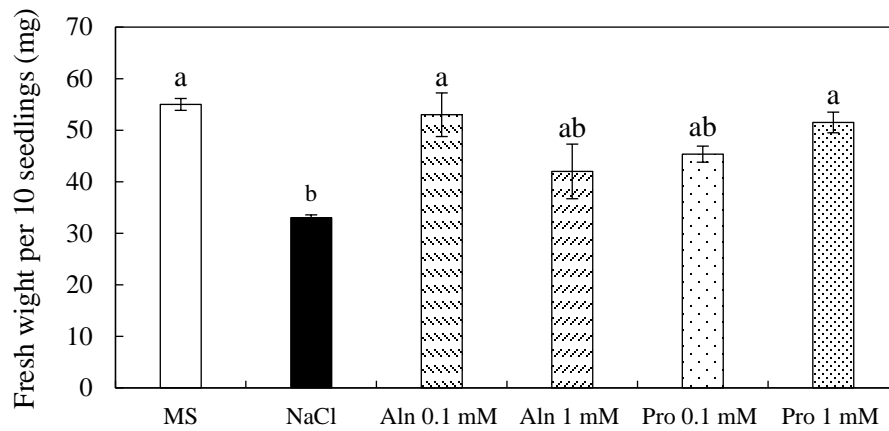
### **3.6.2. Effect of exogenous allantoin when seedlings are transferred to NaCl media**

For the second set of experiments, Col-0 seeds were first allowed to germinate under non-stress condition. When the Col-0 seedlings were 5-days-old, they were transferred to media with 100 mM NaCl or 0 NaCl as control. In this way, the effect of NaCl on the germination of seeds was eliminated and the tolerance of seedlings under different media was directly tested. Figure 3.34 shows a schematic diagram of experimental design and various tested media in this experiment.

After nine days growth under six different media, the beneficial effect of application of 0.1 mM allantoin with 100 mM NaCl was noticeable in 2-week-old seedlings. Fresh weight (Figure 3.35) and primary root length (Figure 3.36) of 2-week-old seedlings in various media were measured and compared. No difference was found between treatments except for 100 mM NaCl. Higher fresh weight and root length was found in seedlings which were grown under control media and 100 mM NaCl with 0.1 mM allantoin or 1 mM proline supplements than the ones on 100 mM NaCl (Figures 3.35 and 3.36). Figure 3.37 shows the phenotype of 2-week-old seedlings that were grown on MS, NaCl, NaCl + 0.1 mM allantoin and NaCl + 1 mM proline media for 9 days. The figure shows that a 0.1 mM allantoin or 1 mM proline supplementation positively affect the phenotype of the seedlings as visually observed root and leaf growth.

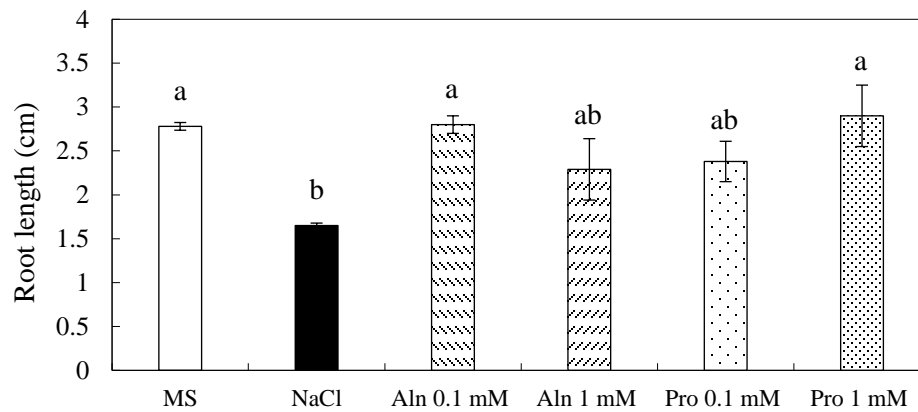


**Figure 3.34. Schematic experimental design to detect the effect of exogenous allantoin on Col-0 seedling growth on NaCl media.** The Col-0 seeds were first germinated on MS media for five days and then were shifted to six different media and were allowed to grow in these media for additional nine days. The six different media were MS media with: 0 NaCl (MS), 100 mM NaCl, 100 mM NaCl + 0.1 mM allantoin, 100 mM NaCl + 1 mM allantoin, 100 mM NaCl + 0.1 mM proline and 100 mM NaCl + 1 mM proline.

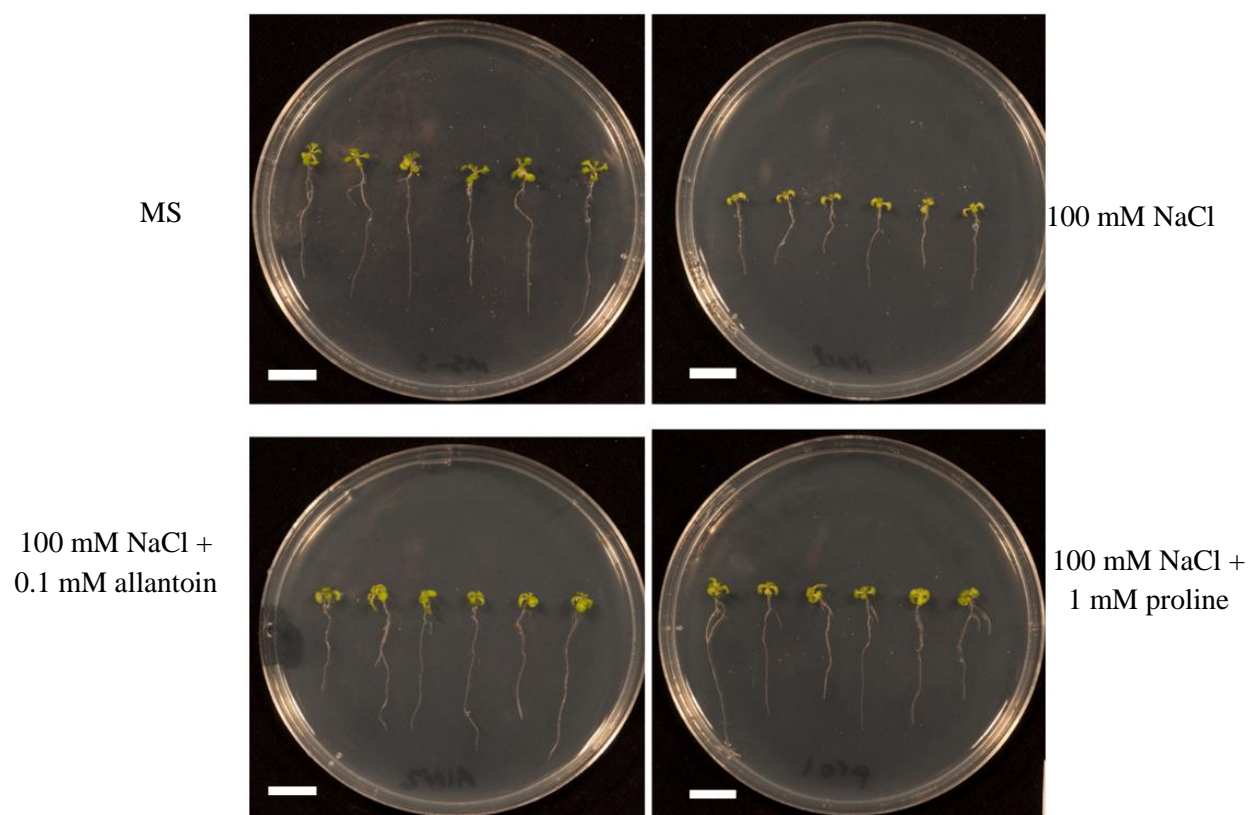


**Figure 3.35. Fresh weight of 2-week-old Col-0 seedlings grown on six different media.** 5-day-old seedlings were grown in six different media for nine days. Data represent the fresh weight as mg per 10 seedlings. MS, media without NaCl; NaCl media containing 100 mM NaCl. Plates containing 0.1 or 1 mM allantoin or 0.1 or 1 mM proline also contained 100 mM NaCl. Measurements were made of at least 30 seedlings per experiment. Data presented are the mean of three independent replicates  $\pm$  SEM. One-way analysis of variance (ANOVA) at ( $p < 0.05$ ) was used to analyze the data which was followed with Tukey's test to compare the means of every treatment to the mean of every other treatment. Bars with the same letter are not significantly different as determined by Tukey HSD test at  $p < 0.05$ . ( $n = 3$ ). Aln: Allantoin; Pro: Proline.





**Figure 3.36. Root length of 2-week-old Col-0 seedlings grown on six different media.** 5-day-old seedlings were grown for nine days in the six different media. MS, media without NaCl; NaCl media containing 100 mM NaCl. Plates containing 0.1 or 1 mM allantoin or 0.1 or 1 mM proline also contained 100 mM NaCl. Measurements were made of at least 30 seedlings per experiment. Data presented are the mean of three independent replicates  $\pm$  SEM. One-way analysis of variance (ANOVA) at ( $p < 0.05$ ) was used to analyze the data which followed by Tukey's test to compare the means of every treatment to the mean of every other treatment. Bars with the same letter are not significantly different (determined by Tukey HSD test at  $p < 0.05$ ). (n= 3). Aln: Allantoin; Pro: Proline.



**Figure 3.37. Phenotype of 2-week-old Col-0 seedlings grown on four different media.** Two-week-old Col-0 seedlings on MS media without NaCl and with 100 mM NaCl, 100 mM NaCl + 0.1 mM allantoin and 100 mM NaCl + 1 mM proline. Seedlings were germinated for five days before being shifted to these media for nine more days before taking the picture. The image is representative of three independent experiments. Scale bar, 1 cm.

## CHAPTER 4. DISCUSSION

The first purpose of this research was to study ureide metabolism under abiotic stress conditions in a non-legume plant, *Arabidopsis*. It was important to clarify if changes in ureide levels are triggered in response to various abiotic stresses in *Arabidopsis*. Some of the questions that were examined here: do ureides increase in *Arabidopsis thaliana* under abiotic stress conditions? Which ureide compound or compounds accumulate in *Arabidopsis* under abiotic stress conditions, and to what level? Do they accumulate in a similar manner in seedlings and adult plants or under different stress conditions? Considering the difference between legume and non-legume plants in the role ureides play in transporting fixed nitrogen, does ureide metabolism in drought-stressed *Arabidopsis* behave similarly to legumes? In this regard, I determined the levels of three ureide compounds in *Arabidopsis* seedlings or leaves under different abiotic stress conditions. In pursuing this work, I hoped to contribute to a better understanding of how ureides are involved in mitigating abiotic stress in *Arabidopsis*, and in plants in general.

### 4.1. Accumulation of allantoin in *Arabidopsis* leaves under drought stress

Accumulation of purine catabolic products, mostly allantoin and allantoate, has been reported in a number of plants (mostly legumes) under water stress conditions. For example, increased levels of both compounds was observed in soybean (*Glycine max*) under water stress (De silva *et al.*, 1996; Purcell *et al.*, 1998; Serraj *et al.*, 1999 and Purcell *et al.*, 2000). Accumulation of allantoin and allantoate has also been reported in common bean (*Phaseolus vulgaris*) under drought stress (Alamillo *et al.*, 2010; Coletto *et al.*, 2014). In fact, in the tropical legumes ureides form 60 to 95% of the organic nitrogen in xylem sap (Christensen and Jochimsen, 1983; Tajima *et al.*, 2004). Therefore, these plants have considerable amounts of ureides even under non-stress conditions. However, accumulation of ureides increases dramatically in tropical legumes under water stress (Todd *et al.*, 2006). As expected, I have shown that *Arabidopsis* did not have a considerable amount of ureides under non-stress conditions. But significant amounts of allantoin was found following prolonged drought in *Arabidopsis* (Figure 3.8). This indicates that accumulation of ureides under prolonged drought is not limited to nitrogen fixing plants or legumes but that it may be a general response in plants. However, only allantoin accumulated in *Arabidopsis* (this is consistent with the upregulation of *AAH* as shown in Figure 3.9), while

legumes tend to accumulate both allantoin and allantoate. Accumulation of these two ureides, usually more allantoate, has been observed in a number of water-stressed leguminous species (De Silva *et al.*, 1996; Purcell *et al.*, 1998; Serraj *et al.*, 1999 and Purcell *et al.*, 2000; Alamillo *et al.*, 2010, Coletto *et al.*, 2014). The difference between *Arabidopsis* and legumes may be related to the high metabolic flux of ureides in transport of fixed N<sub>2</sub> in nitrogen-fixing legumes. Alternatively, it may be indicative of differing flow through the ureide metabolic pathway in response to condition, tissue or development stages in *Arabidopsis*. Particularly since in this research the accumulation of both allantoin and allantoate was only observed in NaCl and mannitol-treated *Arabidopsis* seedlings (Figure 3.24) and not in the leaves of drought- or high light-treated adult plants (Figures 3.8 and 3.17). It would be prudent to test the level of allantoin and allantoate in drought-stressed *Arabidopsis* in different growth stages and also to test the effect of other stresses such as salinity on ureide accumulation in mature plants.

#### **4.2. Accumulation of allantoin in *Arabidopsis* leaves under increased light intensity**

Previous studies indicate that light/dark can affect ureide accumulation. Dark treatment of plants resulted in an accumulation of allantoin and allantoate in *Phaseolus vulgaris* (Engelbrecht, 1955; as cited by Castro *et al.*, 2001), *Funaria hygrometrica* (Hartmann and Arnold, 1974; as cited by Castro *et al.*, 2001) and *Arabidopsis thaliana* (Brychkova *et al.*, 2008). In response to different photoperiods, accumulation of allantoin increased incrementally by photoperiod enhancement in comfrey roots but its accumulation decreased in the underground horizontal stems (rhizomes) (Castro *et al.*, 2001). The authors suggested that their results indicates that in comfrey, roots are the sites of allantoin synthesis while the allantoin in rhizomes is provided by translocation from other organs like roots. Therefore, photoperiod can affect both synthesis of allantoin in roots and the levels of translocation of allantoin to rhizomes (Castro *et al.*, 2001).

Malik *et al.* (2016) reported an increase in the sum of allantoin, allantoate, ureidoglycolate and glyoxylate content under high light (750  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) in *Eutrema salsugineum*. A 2.5-fold increase in total ureide was observed when Yukon ecotype of *Eutrema* was grown under high light, compared to plants grown under same photoperiod and temperature conditions but with moderate light regime (250  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). In the present study, I used the same light intensity, photoperiod and temperature conditions as Malik *et al.* (2016) for *Arabidopsis*, a close, but less stress-tolerant, relative of *E. salsugineum*. When *Arabidopsis* plant were exposed to

increased light condition, accumulation of allantoin in leaves was found a few days after exposing the plants to these conditions (Figure 3.17). However, no detectable allantoin (or uric acid, or allantoate) was observed in plants exposed to moderate light intensity. This might indicate that the accumulation of allantoin in leaves in *Arabidopsis* under high light intensity and long photoperiod was primarily the effect of increased light intensity and not the longer day photoperiod. However, this result does not eliminate the possibility of accumulation of allantoin or other ureides in roots or other tissues of *Arabidopsis* in response to change in photoperiod, since these were not tested.

Vitoria and Mazzafera (1999) reported that in *Coffea arabica* and *Coffea dewevrei*, caffeine can slowly catabolize and form xanthine, and in turn, xanthine converts to ureides. Therefore, following caffeine catabolism, higher levels of allantoin and allantoate were found in these plants (Vitória and Mazzafera, 1999). Pompelli *et al.* (2013) reported that in *Coffea arabica* plants, higher concentrations of allantoin were found under increased light treatment. The seedlings were allowed to grow in two light treatments including 100% solar irradiation (referred to as high light; maximum  $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 50% solar irradiation (referred to as moderate light). The concentration of allantoin in fully expanded young leaves showed a 47% increase in plants under 100% solar irradiation compared to plants under 50% solar irradiation. At first, degradation of the purine alkaloid, caffeine, to xanthine was considered as a source of allantoin in coffee plants. But since the increase in both caffeine and allantoin levels was observed in high light-treated coffee plants, the authors suggested that high levels of allantoin under high light stress might not be related to caffeine degradation (Pompelli *et al.*, 2013). Consistent with results reported by Pompelli *et al.* (2013), I showed that accumulation of allantoin under increased light intensity can be a response to excess light intensity. My research also shows that allantoin accumulates in *Arabidopsis* leaves which is not a high caffeine-containing plant species.

In conclusion, the results reported by Malik *et al.* (2016) suggested that ureides accumulate in *Eutrema* leaf tissue in response to high irradiance and is also associated with higher tolerance of plants to photoinhibition when grown under high light. It was proposed that ureides may contribute to protection of plants from photooxidative stress (Malik *et al.*, 2016). My research on the effect of increased light intensity on ureide concentration provides a new insight into the contribution of a particular ureide compound, allantoin, in protection of plants in response to high light.

#### **4.3. Enhanced levels of allantoin and allantoate in Arabidopsis seedlings induced by NaCl and mannitol treatments**

Salinity is one of the most important abiotic stresses that negatively effects rice (*Oryza sativa*) yield in most regions (Ondrasek *et al.*, 2011). Therefore, recently there have been more studies focusing on metabolomic profiling or transcriptome comparison of salt-stressed rice or salt tolerant rice ecotypes in different developmental stages. In a number of these studies, higher concentrations of allantoin were found in salt-stressed rice. For example, metabolic profiling analysis showed that allantoin is one of the principal compounds that is enhanced in rice roots in response to a long term mild NaCl stress (Nam *et al.*, 2015). However, the function and source of this compound in salt-stressed rice roots remains unknown (Nam *et al.*, 2015). In the present research, I showed that allantoin, along with allantoate, increases in Arabidopsis seedlings that were treated with NaCl, compared to control non-treated seedlings (Figure 3.24). The source of these compounds is still unclear, but a decrease in expression of *ALN* and *AAH* with NaCl treatment was observed by RT-PCR analysis (Figure 3.25). More recently, Lescano *et al.* (2016) reported that allantoin accumulates under salt stress in Arabidopsis and its accumulation is mediated by downregulation of *ALN*. This report and my results support the effect of NaCl on ureides level regulated at the transcription level in Arabidopsis seedlings.

Wang *et al.* (2016) reported that in response to NaCl treatment of rice seedlings, the concentration of allantoin was found to be 42.6-fold higher in roots of a salt-tolerant rice genotype compared to a salt-sensitive genotype. Thus, Wang *et al.*, (2016) suggested that it is possible that an accumulation of allantoin directly contributes to the salt tolerance of salt-tolerant rice genotypes. In addition to rice, irrigation of *Crithmum maritimum* plants with 100 mM NaCl increased allantoin and allantoate levels by 3.5 and 2.3 fold, respectively (Ventura *et al.*, 2014). *Crithmum maritimum* is a salt-tolerant plant species (a halophyte) that is well known for high levels of antioxidants (Meot-Duros and Magne, 2009; Ventura *et al.*, 2014). Altogether, my results also propose that allantoin and allantoate may be critical metabolites in response to NaCl stress in Arabidopsis seedlings.

Osmotic stress is imposed by drought, freezing and salinity stresses. Osmotic stress resulting from any of these abiotic stresses has a serious negative impact on plant growth (Xiong and Zhu, 2002). In the present reseach, I used mannitol to test the effect of osmotic stress on ureide accumulation in Arabidopsis seedlings. By exposing seedlings to osmotic stress induced by

mannitol, I found enhanced levels of allantoin and allantoate in the seedlings compared to control plants (Figure 3.24). Following mannitol treatment, consistent with elevated ureide level, expression of *ALN* in the seedlings reduced (Figure 3.25). These results are in line with a report by Watanabe *et al.* (2014a) of a significant increase of allantoin when *Arabidopsis* seeds were germinated and grown on media containing 150 and 200 mM mannitol. In addition, *Ataln* mutants with a constitutive high allantoin accumulation exhibited more osmotic stress tolerance and better growth in the presence of 100, 150 and 200 mM mannitol compared to wild-type (Watanabe *et al.*, 2014a). Taken together, the results of Watanabe *et al.* (2014a) and my observations underline the importance of allantoin in plant osmotic stress tolerance and the potential function of this metabolite in plant response to osmotic stress.

Moreover, interestingly, I observed that no ureides are detectable in seedlings exposed to NaCl and mannitol when tested between two and 10 hours of exposure. This might suggest that ureide accumulation is not involved in a short-term response to these stresses in *Arabidopsis* seedlings and their accumulation contributes to the moderate to long term response to stress.

#### **4.4. Expression of ureide metabolism genes under abiotic stress conditions**

Brychkova *et al.* (2008) reported that transcript levels of ureide synthesis genes (*XDHI*, *UO*) were enhanced in *Arabidopsis* under dark stress while transcripts of the ureide catabolism genes (*ALN* and *AAH*) were decreased. Transcript level of both *ALN* and *AAH* increased on day 1 of dark treatment but then continuously decreased up to day 6. After day 6, the plants were transferred to a 16 h light/8 h dark regime for recovery. Transcripts of *ALN* and *AAH* started to increase when tested three days after recovery. Watanabe *et al.* (2014a) subjected 18-day-old wild-type *Arabidopsis* plants to 24-hour drought shock. They found that the expression of *XDHI* and *UO* reached the maximum at hour 8 of the drought shock. Afterward, gene expression dropped steadily up to hour 24 of the shock treatment. Understanding ureide gene expression pattern under abiotic stress treatments was one of my goals for this part of the research. Some questions to be answered included: how the expression of ureide genes changes under abiotic stress conditions? Is accumulation of ureides under stress associated with alteration in expression of their biosynthesis and catabolism enzyme genes?

In this study, the expression of ureide genes was tested in *Arabidopsis* leaves under drought and increased light intensity stresses. Under drought, an increase in expression of *UO* preceded

the other ureide genes, with a concurrent increase in levels of allantoin (Figure 3.9). Up-regulation of most ureide metabolism enzyme genes in response to withholding water on day 10 may be a general function related to the role of ureide metabolism in remobilizing nitrogen, but the lack of induction of *ALN* transcript level on day 10 led to the specific accumulation of allantoin (Figure 3.9). This may also be a specific mechanism to induce allantoin accumulation, in response to upregulation of the other ureide metabolic genes. If so, this may have evolved after the mechanism that upregulates the entire pathway, as a specific mechanism to increase allantoin concentration. The observation of a wilting phenotype after 10 days of drought (Figure 3.12) may support the involvement of ureide gene expression in recycling nitrogen. However, while some cell death may have occurred (leading to a production of purine intermediates), most of the tissue recovered following re-watering, suggesting remobilization of nitrogen within the cell, rather than transport of nitrogen from senescing tissues.

The transcript levels of *UO* and *ALN* were also measured in drought-stressed and untreated leaf samples using qRT-PCR. The dramatic changes in transcript levels of *UO* following withholding of water suggests the importance of *UO* function under stress (Figure 3.10). Although, the activity of *UO* and *ALN* was not tested, the increase in *UO* expression without a corresponding change in *ALN* expression can explain allantoin accumulation under stress under drought and possibly other stress conditions. Since significant urate stores have not been reported in *Arabidopsis* leaves routinely or under abiotic stress, therefore, either an increase of *XDH* transcription, post-translational activation of *XDH* enzyme activity, or both, would be expected to accompany an increase in *UO* transcription to provide urate as a substrate. Similarly to drought stress, the increase in the expression of *UO* with no change in expression of *ALN* led to the accumulation of allantoin in *Arabidopsis* leaves under increased light stress (Figure 3.18). Lower expression of *ALN* under both NaCl and mannitol treatments and *AAH* mainly under NaCl stress was also observed in the seedlings (Figure 3.25).

Overall, my results show that increased levels of allantoin in response to stress in *Arabidopsis* is regulated (at least to some degree) by an induction of *UO* gene expression and possibly partly through a reduction of allantoin degradation to allantoate by *ALN*. Furthermore, it can be concluded that different abiotic stress conditions influence the expression of ureide metabolism genes. This effect mainly included the expression of *UO* in stressed-leaves and the expression of *ALN* and *AAH* in stressed-seedlings. The difference between developmental stage of



adult plants and seedlings might be an explanation for this observation. Although, there has not been any specific report on difference of ureide accumulation or expression pattern during plant development and growth, I consistently found more allantoin accumulation in adult plants' leaf tissue than seedlings in my research. Lescano *et al.* (2016) suggested that the difference between accumulation or expression pattern of allantoin and allantoate in *Arabidopsis* under various abiotic stresses may result from the different impact of physiological stress and senescence on regulation of purine catabolism

#### **4.5. *Arabidopsis* ureide mutants and their performance under abiotic stress**

In a parallel approach, homozygous T-DNA knock-out lines were used to address the question of whether mutants impaired in ureide metabolism were more susceptible or tolerant to abiotic stress conditions. The hypothesis was if a ureide compound is important in protecting plants in response to stress, its constitutive accumulation or impairment in its production might improve or decrease plant performance under stress, respectively.

I examined four *Arabidopsis* knockout mutants of the ureide metabolism pathway, *xdh1*, *aln*, *aah* and *uah*. *Arabidopsis xdh1* mutant is impaired in a gene upstream of allantoin while *aah* and *uah* mutants are impaired in downstream metabolism of allantoin. I found that the *ALN*-knockout mutant (*aln-3*) with elevated levels of allantoin, but not the other three mutants, showed an enhanced stress tolerance under increased light condition (Figure 3.6). Similarly, under drought and NaCl stresses, *aln-3* exhibited higher stress tolerance compared to the Col-0. Since *xdh1*, *aah* and *uah* plants did not exhibit stress tolerance phenotype, this may indicate that the stress tolerance phenotype of *aln-3* is a result of allantoin accumulation. If the stress tolerance of *aln-3* was the result of an overall inhibition of ureide metabolism, then the other mutants should probably show the same stress tolerance response.

Although comparative transcript profiling of wild-type and *aln* mutant seedlings grown under standard conditions using microarray analysis demonstrated altered transcription patterns in several genes (Watanabe *et al.*, 2014a), there has not been any report of altered visual phenotypic characteristics exhibited by allantoinase-negative plants. Under standard *Arabidopsis* growth conditions (Watanabe *et al.*, 2014a) and with altered nitrogen sources (Yang and Han, 2004; Werner *et al.*, 2008), *aln* growth has been similar to wild-type. In my study, *aln-3* mutant also did not show a visual phenotype difference from wild-type under non-stress condition. However,

increased tolerance of *aln-1* exposed to a one hour air-drying dehydration shock or mannitol/PEG osmotic stresses has been reported in Arabidopsis (Watanabe *et al.*, 2014a).

On the other hand, some findings showed that loss of gene function in enzymes upstream of ALN, including *UO* and *XDH*, can cause negative phenotypic changes. Hauck *et al.* (2014) reported that Arabidopsis *uo* mutants which accumulated uric acid exhibited low germination rate and were unable to develop to adult plants in the absence of supplemental sucrose in the media. Sucrose helped the mutant seedlings to develop roots and green cotyledons. As peroxisomes (glyoxysomes) were found to be defective in the *uo* mutants, it was suggested that this explains the need for external sucrose to serve as source of energy and carbon for seedlings to develop (Hauck *et al.*, 2014). The mutants became independent from sucrose later in the growth stage and they were phenotypically similar to wild-type when tested under non-stress condition (Hauck *et al.*, 2014). However, the response of *UO* mutants to the stress conditions has not been studied. Nakagawa *et al.* (2007) reported that *Atxdh1* and *Atxdh2* RNAi transgenic plants that accumulated xanthine exhibited severe growth and reproductive deficiency and advanced leaf senescence under normal growth conditions. Moreover, accelerated chlorophyll degradation and a higher number of dead cells were found in *Atxdh1* mutants under stress, probably due to the lack of allantoin and allantoate in the mutants (Brychkova *et al.*, 2008). Under drought shock, *Atxdh1* and *Atxdh2* RNAi knockdown seedlings accumulated less proline, a cellular protectant, and showed lower mRNA levels of the encoding gene for the proline biosynthesis enzyme (*P5CS1*) (Watanabe *et al.*, 2014b). It was mentioned by Brychkova *et al.* (2008) and Watanabe *et al.* (2010) that the stress sensitive phenotype of *xdh1* might be related to the lack of downstream compound(s) of the ureide metabolism pathway in this mutant. Overall, these results suggest that downstream compounds of ureide pathway such as allantoin and allantoate may effectively protect plants from the negative effects of the various abiotic stresses.

In the present study, the improved stress tolerance in both seedlings and adult *aln-3* mutants compared with wild-type suggests that an accumulation of allantoin increases stress tolerance throughout the vegetative growth period. The enhanced tolerance of *aln-3* to drought stress was associated with increased fresh/dry weight (Figure 3.13), chlorophyll, and photochemical efficiency of PSII (Figure 3.15) compared to wild-type. The *aln-3* plants also exhibited better performance under increased light conditions as shown by higher rates of chlorophyll and photochemical efficiency of PSII (Figure 3.22). In seedlings, the higher tolerance of *aln-3* mutants

was significant in parameters such as root length and fresh weight under NaCl stress (Figures 3.27 and 3.29). As allantoin metabolism is part of nitrogen re-mobilization in plants, negative effects of the loss of ALN function in *aln-3* plants under non-stress conditions should be considered. However, in my research, I did not observe any visual negative effect of lack of expression of *ALN* in *aln-3* plants. Interestingly, no negative effect was observed for *aln-1* and *aln-2* by Watanabe *et al.* (2014a). It is not clear if loss of ALN function in *aln* mutants is compensated for some other metabolic mechanism or the loss of ALN in the mutants is not phenotypically visible, at least under the growth conditions I employed. Both *aln-3* seedlings and adult plants were provided with nitrogen either in their MS media, or their irrigation water (as fertilizer), during the experiments.

Results presented here are the outcome of using a different *aln* line as reported by Watanabe *et al.* (2014a), and in response to different stress treatments and vegetative stages. This indicates that constitutively high levels of allantoin in *aln* mutants leads to the better response of *Arabidopsis* under abiotic stress and suggests that allantoin might confer a plant stress tolerance during growth and development from seedling to adult stage.

#### **4.6. Effect of exogenous allantoin on increasing the tolerance of seedlings to NaCl stress**

The overall results of this study suggest that the ureide compound, allantoin, confers plant tolerance under abiotic stress conditions. However, another question might be: can an exogenous application of allantoin increase plant stress tolerance? The hypothesis that allantoin can improve plant tolerance to abiotic stress (here NaCl stress) was tested by analysing the influence of exogenous allantoin on the performance of *Arabidopsis* seedlings under NaCl stress.

Watanabe *et al.* (2014a) tested the effect of an exogenous application of allantoin on expression of typical stress marker genes (*RD29A*, *RD29B* and *RD26*) in wild-type *Arabidopsis* seedlings under non-stress condition. The expression of these genes was induced with 100 and 1000  $\mu$ M exogenous allantoin treatment of the seedlings. Furthermore, transcript levels of *NCED3*, a gene that encodes a key ABA biosynthesis enzyme, was increased by application of 1000  $\mu$ M exogenous allantoin (Watanabe *et al.*, 2014a). Based on these results, I selected the same concentrations of allantoin (0.1 mM and 1 mM) to test the effect of exogenous allantoin under NaCl.

I tested the effect of exogenous applications of allantoin on Col-0 seedling tolerance under 100 mM NaCl stress in two sets of experiments as described in section 3.6. Reduction of plant

growth is a common effect of salinity stress (Parihar *et al.*, 2015). In my experiments, the NaCl treatment of *Arabidopsis* Col-0 seedlings led to a lower fresh mass and root length compared to no NaCl treatment. However, an exogenous application of 0.1 mM allantoin enhanced seedling mass and root length when grown under 100 mM NaCl (Figures 3.30 and 3.31) or when exposed to 100 mM NaCl compared to only NaCl media (Figures 3.35 and 3.36). Although, an application of 1 mM allantoin did not show any noticeable beneficial effect on seedlings' performance, it also did not have a negative effect compared to the media with only NaCl. These overall results indicate that allantoin has a positive effect on seedling tolerance to NaCl stress.

Accumulation of the amino acid proline in higher plants has been reported frequently under various environmental stresses (Debnath *et al.*, 2011). Proline is an osmolyte compound with the main function of osmotic adjustment under stress condition such as salinity. Proline can also play other roles under stress such as stabilizing enzymes and cellular structures and scavenging ROS (Ashraf and Foolad, 2007). Application of exogenous proline has been frequently reported to improve plant salt-tolerance (Khedr *et al.*, 2003; Hoque *et al.*, 2007; Hoque *et al.*, 2008; Sobahan *et al.*, 2009; Ben Ahmed *et al.*, 2010; Nounjan *et al.*, 2012). Therefore, I used the same concentrations of proline as allantoin to have a control compound for seedling performance under NaCl and exogenous allantoin.

In my experiments, generally exogenous allantoin increased seedling tolerance at 0.1 mM concentration, while proline was more effective on 1 mM. The difference between the effective concentrations of these two compounds probably is related to the variation in their mechanism or function under NaCl stress. The preliminary results in this part suggest that unlike proline, that has an osmoprotectant function, allantoin might be involved in a signalling mechanism. This may explain the effect of allantoin on NaCl stress tolerance at 10 fold less concentration compared to the effective concentration of proline. Altogether, this speculation is based on my observation on allantoin having a positive effect on seedling tolerance at lower concentration (compared to proline), it is also supported by the results of Watanabe *et al.* (2014) and Takagi *et al.* (2016) on the effect of allantoin on activation of ABA and jasmonate metabolism. However, I did not measure allantoin or proline levels in seedlings under different treatments. Clearly more experiments need to be performed to explain the function of exogenous allantoin on enhancing NaCl tolerance and the difference between the mechanism of exogenous allantoin and proline. In the meanwhile, the current results indicate the positive effect of a concentration of allantoin on

seedling stress tolerance as already was established for the well-known stress compound, proline. More investigations on the effect of exogenous application of allantoin on adult plant tolerance and the plant's metabolic response to this compound under abiotic stress could provide more applicable advantages.

#### 4.7. Allantoin

Historically, in plants, the most documented information about allantoin is from legumes. Allantoin had been primarily studied along with allantoate as important compounds in nitrogen storage and translocation. However, during the course of my work, the research on abiotic stress, especially profiling of stress-inducible metabolites in plant tissues and the metabolic analysis of stress-tolerant plants (Oliver *et al.*, 2011; Yobi *et al.*, 2013; Degenkolbe *et al.*, 2013; Nam *et al.*, 2015), has increased attention on allantoin in plants more than ever. The potential function of allantoin in response to dehydration has been suggested in a number of global metabolic and biochemical studies of dessication-tolerant plants (Oliver *et al.*, 2011; Yobi *et al.*, 2013). In both of these studies, allantoin concentration was more abundant in the *Sporobolus stapfianus* (Oliver *et al.*, 2011) and *Selaginella lepidophylla* (Yobi *et al.*, 2013) under dehydration stress. High levels of allantoin have been observed in tolerant populations of rice cultivars under drought. Allantoin and other metabolites (e.g. galactaric, gluconic acid and salicylic acid glucopyranoside) identified by metabolite profiling have been proposed as promising marker candidates for drought tolerance (Degenkolbe *et al.*, 2013). Allantoin was suggested as a metabolic marker of salt stress since it was accumulated in all 38 tested rice genotypes in response to long term salinity stress (Nam *et al.*, 2015).

Wang *et al.* (2007) reported the possibility of allantoin release from rice roots to the rhizosphere and its interaction with the soil bacteria and other organisms. It was not clear if the effect of allantoin on the rhizosphere would be related to nitrogen metabolism or an unknown signalling process. Additionally, exogenous applications of allantoin in rice soil increased microbial diversity as well as microbial biomass carbon (Wang *et al.*, 2010b). However, it is still to be determined if an exudation of allantoin from roots into the soil or an application of allantoin results in a significant advantage for rice (Wang *et al.*, 2010b).

Recently, higher levels of allantoin has been reported under nitrogen deficiency (Pompelli *et al.*, 2013) and phosphorus limitation (Pant *et al.*, 2015) in coffee and Arabidopsis plants,

respectively. Enhanced levels of allantoin has been found in *Arabidopsis* seedlings in response to air-drying and osmotic stresses (Watanabe *et al.*, 2014a). Rose *et al.* (2012) reported that in response to zinc deficiency and excess amount of bicarbonate, nitrogen rich metabolites including allantoin accumulated in rice roots.

The results of the present study demonstrated enhanced levels of allantoin in adult *Arabidopsis* plants under drought and increased light intensity stresses and in seedlings under NaCl and mannitol treatments. Moreover, an exogenous application of allantoin significantly improved plant performance under NaCl stress. Generally, my results confirm that allantoin has a potential role(s) in response to abiotic stress in plants but since the clarification of function of allantoin was not the focus of this research, I have not studied the molecular and cellular aspects of allantoin mechanism in details. It also seems that due to the limited knowledge on allantoin's involvement under abiotic stress response especially until recently, the potential function of allantoin under stress has not been explicitly studied in most of the reports on allantoin. In the next section, I have collected the available information and suggestions on the function of allantoin under abiotic stress conditions in plants.

#### **4.7.1. The function of allantoin under abiotic stress**

The mechanism behind allantoin as a stress protectant is still a controversial topic. While some reports have proposed that allantoin functions as an antioxidant (Gus'kov *et al.*, 2004; Brychkova *et al.*, 2008), other studies have suggested that allantoin functions under stress response with a non-antioxidant role (Wang *et al.*, 2012, Watanabe *et al.*, 2014a). To date, the two main hypothesis for allantoin's role under abiotic stress include influencing ABA signaling and/or antioxidant activity.

The phytohormone ABA plays important roles in plant responses to abiotic stresses such as induction of production of ROS (Jiang and Zhang, 2002) and enhancement of the activity of enzymatic and non-enzymatic antioxidants (Jiang and Zhang, 2004). The interest in a possible link between the ureide pathway and ABA was principally developed in a report by Yesbergerova *et al.* (2005). The authors reported that an increase in the activity of XDH in tomato parallel the increase in ABA level under water stress. Moreover, the expression of both *XDHI* and *XDH2* (more significantly *XDHI*) was induced in response to an exogenous application of ABA. (Yesbergerova *et al.*, 2005). Later, in 2010, Alamillo *et al.* reported the effect of an application of

exogenous ABA on transcript levels of *UO*, *ALN* and *AAH* in leaf and shoot tissues of common bean in the absence of stress. The expression of all three genes was significantly induced compared to untreated control samples. Based on these observations, an effect of ABA on the expression of ureide genes was suggested to be a mechanism to trigger leaf senescence in order to recycle enough nitrogen for the rapid formation of seeds (Alamillo *et al.*, 2010). However, Watanabe *et al.* (2014a) reported that the interaction of ABA and ureide metabolism is related to abiotic stress tolerance in *Arabidopsis* rather than recycling nitrogen. ABA is synthesized through a complex *de novo* pathway in plants. The cellular level of ABA can also be determined by ABA conjugation and de-conjugation reactions (Nambara and Marion-Poll, 2005). ABA conjugation refers to the inactivation of ABA by conjugation to another molecule, most commonly glucosyl ester. During ABA de-conjugation, hydrolysis of the conjugated ABA results in a free pool of ABA in cells (Lee *et al.*, 2006). The results of Watanabe *et al.* (2014a) showed that both ABA *de novo* biosynthesis and the de-conjugation of ABA glucose esters were activated in *aln* mutants under non stress conditions. Moreover, even without stress treatment, an application of exogenous allantoin to wild-type *Arabidopsis* seedlings resulted in the activation of both ABA production steps as well as higher levels of ABA. Altogether, Watanabe *et al.* (2014a) suggested that allantoin functions in the abiotic stress response by an activation of ABA metabolism in *Arabidopsis* and the significantly higher accumulation of ABA in the *aln-1* mutant might be the source of the tolerant phenotype of this mutant under stress. Most recently, the role of allantoin in activation of the jasmonate signaling pathway probably through an ABA-dependent mechanism was suggested (Takagi *et al.*, 2016). The authors reported that in *aln-1* mutants of *Arabidopsis*, expression of ABA- and jasmonate-responsive genes is upregulated compared to wild-type plants under non-stress condition. They also found that an exogenous application of allantoin induced the expression of jasmonate responsive genes in wild-type plants suggesting that allantoin might be involved in stress signaling and hormone homeostasis and interaction (Takagi *et al.*, 2016). Further examinations would throw light on physiological functions of allantoin and its potential relationship with other ureides under stress.

On the other hand, like many of the abiotic stresses, prolonged drought stress is associated with impaired ROS balance and accumulation of ROS (Gechev *et al.*, 2006; Petrov *et al.*, 2015). Excess generation and accumulation of ROS during stress can result in oxidative damage and an induction of cell death (Mittler, 2002; Golldack *et al.*, 2014). In the present research, lower

concentrations of  $O_2^-$  and  $H_2O_2$  were found in drought-stressed *aln-3* leaves than in wild-type leaves (Figure 3.16). Allantoin and allantoate were previously identified to act as cell ROS protectants in response to senescence since their presence decreased ROS accumulation in wild-type *Arabidopsis* after dark treatment (Brychkova *et al.*, 2008). It is been reported that a deficiency in uric acid and downstream metabolites of uric acid in an *Arabidopsis XDH*-mutant under drought shock resulted in an accumulation of a high concentration of  $H_2O_2$  in the mutant seedlings compared to wild-type (Watanabe *et al.*, 2010). An application of exogenous uric acid elevated drought shock tolerance in *XDH*-suppressed *Arabidopsis* seedlings suggesting that the intermediates of ureide metabolism might provide the antioxidative potential required for protection of the plant (Watanabe *et al.*, 2010).

Gus'kov *et al.* (2001) reported that allantoin may be able to modulate or induce the activity of antioxidant enzymes under oxidative stress conditions. Pre-treatment with allantoin significantly increased the activity of superoxide dismutase and catalase in various tissues of rats exposed to high oxygen pressure compared to controls. Furthermore, pre-treatment of onion (*Allium cepa* L.) seedlings with allantoin before  $H_2O_2$  treatment reduced the levels of chromosomal aberrations (Gus'kov *et al.*, 2004). Wang *et al.* (2012) reported that levels of allantoin in rice grains showed a positive correlation with seedling survival under low temperature and drought stress but allantoin did not show an antioxidant function. It is well known that uric acid acts as an antioxidant in human plasma, protecting cells by scavenging harmful radicals (Yeum *et al.*, 2004, Hediger *et al.*, 2005). However, excess amounts of uric acid have been found to be toxic in both plant (Hauck *et al.*, 2014) and human (Sautin and Johnson, 2008) cells. To date, no toxic effect has been reported for high levels of allantoin in *ALN*-mutants of *Arabidopsis* which accumulate allantoin. Gus'kov *et al.* (2004) reported that a comparison of the effects of different levels of allantoin on its antimutagenic activity indicated that allantoin did not display cytotoxic or cytostatic effects at high concentrations (up to  $10^{-2}$  M) in onion seedlings.

Overall, based on these reports, although allantoin has shown antioxidative effects or has reduced ROS levels in plant tissues, there has been no reports on *in vitro* antioxidant activity of allantoin. In an experiment done in the Todd lab, exogenous  $H_2O_2$  was applied to solutions with or without soybean leaf discs and with or without allantoin. The ROS amount did not show a significant change in solutions containing exogenous allantoin without leaf discs. However, pre-treatment of leaf discs with allantoin decreased the exogenous  $H_2O_2$  level, indicating the indirect



effect of allantoin on reducing ROS levels. The requirement of the leaf discs for allantoin to be effective suggests that this effect is a cellular process such as up-regulation of cell ROS detoxification activities (Jodi Souter, U of S PhD Thesis). Therefore, the indirect function of allantoin in reducing ROS under stress rather than a direct antioxidant activity may provide the explanation for all of the observations on allantoin antioxidant activity to date. However, in the case of plants, it is still not clear whether allantoin modulates the ROS scavenging process directly or indirectly through ABA production and the effects of ABA in response to stress in plants. Accumulation of ABA under stress can increase ROS generation whereas it also upregulates the transcript of antioxidant enzymes and elevates the activity of antioxidant enzymes (Jiang and Zhang, 2002). Moreover, allantoin might play additional unknown roles during abiotic stress in plants in order to mediate ROS levels.

#### **4.8. Principle questions addressed in this thesis and anticipated significance of the research**

My research provides an update on ureide metabolism in plants under a number of abiotic stresses. To date, all of the work regarding ureide metabolism under prolonged drought has been done in legumes, considering the importance of ureide metabolism in these plants (transport of fixed nitrogen). This work provides the first report on ureide level and expression of ureide metabolic genes in a non-legume plant under prolonged drought. It also indicates a common molecular mechanism in induction of *UO* expression, that probably contributes to the accumulation of allantoin in *Arabidopsis* under drought and increased light intensity. Aside from extended drought, this is the first report on ureide content and gene expression in *Arabidopsis* seedlings exposed to NaCl as well as the effect of exogenous allantoin on seedling tolerance to NaCl. Overall, the combination of experiments on drought and increased light in adult plants, and NaCl and mannitol treatments in seedlings, have identified potential common regulatory mechanisms. Based on all of my observations, I have developed a model to explain the ureide metabolism response to abiotic stress conditions in *Arabidopsis*.

As depicted in Figure 4.1, in response to abiotic stress, urides (mainly allantoin) accumulate in plant cells. Simultaneously, the transcription of *UO*, which encodes the enzyme uricase involved in allantoin production, is up-regulated early after abiotic stress. It seems that *UO* is the first gene in the pathway, the expression of which is affected by stress conditions. While,

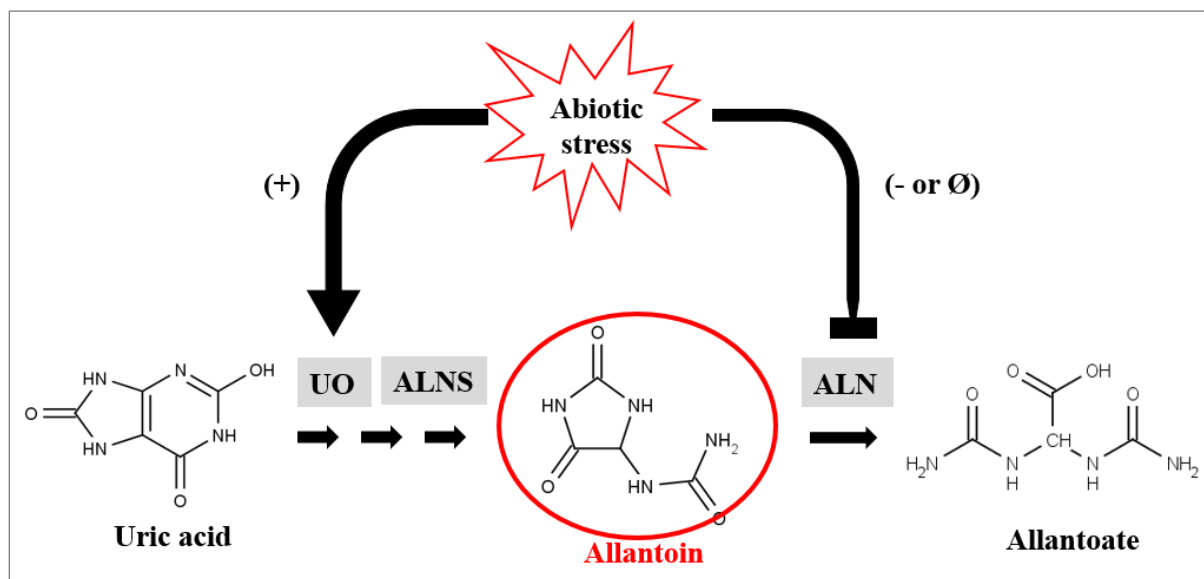
transcription of gene(s) coding for enzymes involved in the degradation of accumulated ureide compound(s) do not show a change or are decreased.

Early in my thesis work, to investigate the possible effect of ureide gene mutations in a plant's response to stress, a number of T-DNA insertion mutants defective in various steps of the ureide metabolism pathway were used. Based on preliminary results from phenotypic comparisons of a number of these mutants and the initial observation of allantoin accumulation in wild-type plants under stress, *aln* was selected for more detailed investigation. The *ALN*-knockout mutation in *aln-3* resulted in an elevated amount of allantoin and increased the stress tolerance in the mutant in response to various abiotic stress conditions.

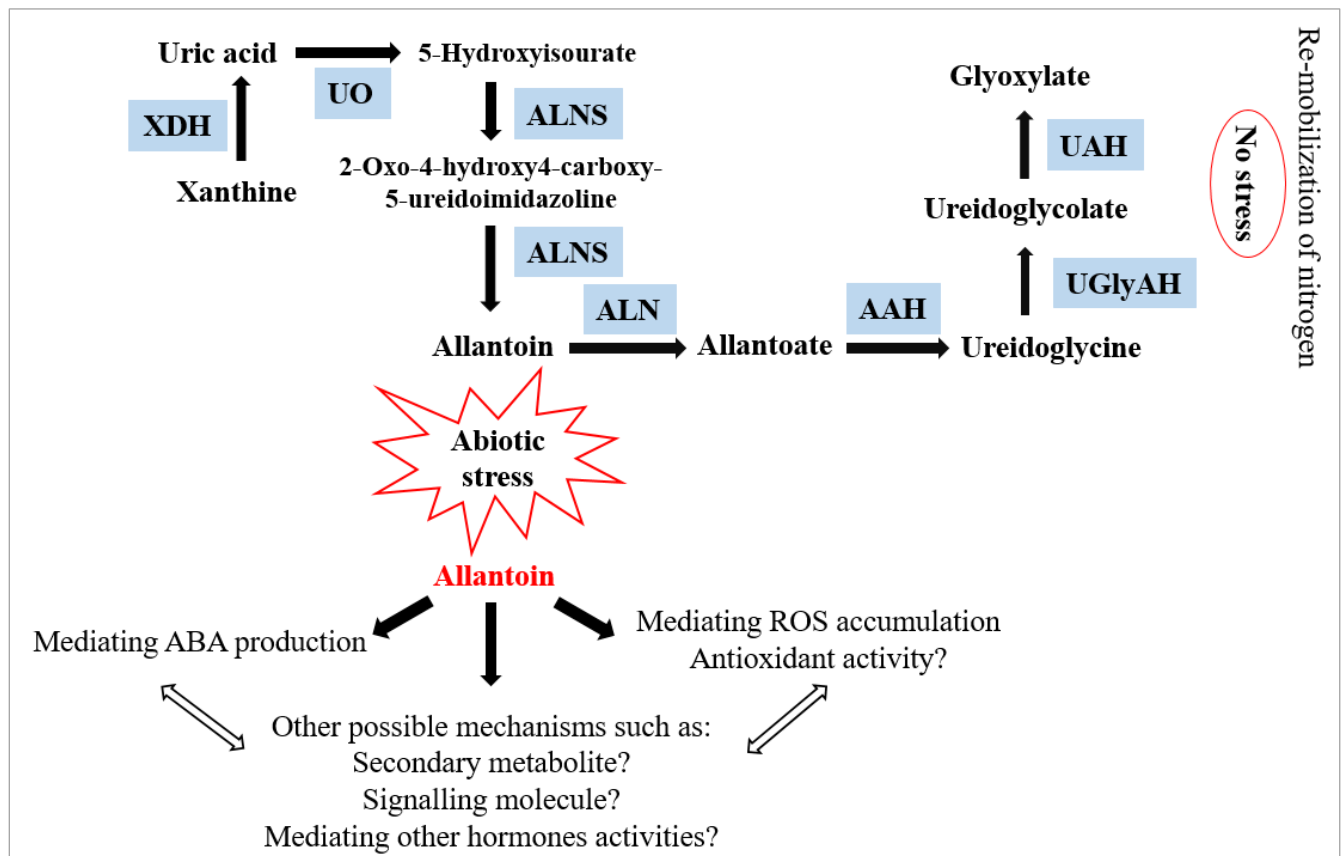
The results of this research indicate an involvement of the ureide pathway under stress is mostly through the synthesis and accumulation of allantoin which improves the performance of *aln* mutant plants under stress when accumulated at high levels. Under non-stress condition, the ureide metabolism pathway functions in recycling nitrogen from purines but in response to stress condition the function of the pathway is not limited to nitrogen re-mobilization. Accumulation of allantoin in cells might have different impacts on plant stress tolerance and might play various roles that still need to be clarified. Based on results of this research and current knowledge on allantoin, I formed a model proposing a putative role or roles for allantoin in plants under abiotic stress (Figure 4.2).

The questions which were addressed here can be used for further investigation of ureide metabolism and accumulation in stress-tolerant plant species from different genera. More importantly, the results obtained here will provide an insight into the role of urides in important crops under stress. Further studies will help to ascertain the function of ureide accumulation and could be useful in future breeding programmes to improve the abiotic stress tolerance in crops. Finally, the tools I have provided to analyze the promoter::reporter and overexpression lines can be incorporated to increase our knowledge on ureide accumulation or function in Arabidopsis.

Altogether, the information provided by this research can help and encourage plant scientists to pursue additional investigation of ureides (including allantoin) and their role in mediating abiotic and potentially biotic stress response in plants.



**Figure 4.1. Proposed model depicting the effect of abiotic stress on ureide metabolism.** Abiotic stress conditions positively affects the expression of *UO* relatively early in response to stress, but has a negative effect or no effect on the expression of *ALN*. Therefore, allantoin level increases under abiotic stress in *Arabidopsis*. *UO*; Urate Oxidase. *ALNS*; Allantoin Synthase. *ALN*; Allantoinase.



**Figure 4.2. Proposed model for the possible role(s) of allantoin in Arabidopsis under abiotic stress.**

Under non-stress condition, allantoin, as part of the ureide metabolic pathway, helps the plant to recycle nitrogen from purine compounds. Under stress condition, the level of allantoin increases in the plant to aid the plant in coping with unfavourable condition. Allantoin can increase plant stress tolerance by mediating the production of ABA or mediating the amount of ROS. However, apart from these roles, allantoin may contribute to plant stress tolerance by other functions that needed to be elucidated, and may interact with each other.

#### 4.9. Remaining questions

The results obtained in this study in association with other observations on ureide accumulation under abiotic stress can be useful agriculturally. However, once the main questions of this research are answered, more questions that need to be addressed include: how will ureide enzyme genes be expressed in specific tissues or morphological stages change in response to abiotic stress? Will expression show coincident increase with accumulation of ROS in these tissues? In order to answer these question, a number of Arabidopsis ureide promoter-reporter lines will be constructed using reporter genes (e.g. *GFP*). Then, under controlled environmental conditions, the proteins will be localized using enzymes with *GFP* or *GUS* fusion under the control of their own promoters. The other main question is: how will transgenic plants overexpressing ureide genes respond to abiotic stress conditions compared to wild-type plants? To answer this question, a number of ureide overexpression lines (using CaMV 35S promoter) will be generated and will be used in abiotic stress experiments. In order to answer these questions, I have generated two ureide transgenic plants (including *XDHI* promoter::reporter and *UO* overexpressed plants) and some of the tools to develop the transgenic Arabidopsis lines. The procedures to generate vector constructs with a number of ureide promoters and coding genes and subsequent transgenic plants are described in Appendix A. These materials will be completed or used by other researchers in our group (or other groups) to answer the questions in this section.

Other remaining questions include: Is ABA mediation the only function of allantoin under abiotic stress or is it involved in protecting plants by other mechanism(s), such as regulation of other hormones? Where exactly in the cell does allantoin accumulate and function in response to stress? Allantoin is formed in the peroxisome and then translocated through the cytosol to the endoplasmic reticulum where it is converted to allantoate by allantoinase (Werner and Witte, 2011). In addition to *de novo* ABA synthesis in plastids, it is likely that ABA increases in response to abiotic stress results from de-conjugation of ABA-glycosides through the action of ER-localized  $\beta$ -glucosidases (Watanabe *et al.*, 2014a). Thus, co-localization of allantoinase and these  $\beta$ -glucosidases in the ER may explain how and when allantoin influences ABA production in response to stress.

As part of this reseach, my focus has been toward understanding the molecular aspect of ureide accumulation. However, for more definite conclusion about the regulation of ureide

accumulation, detecting the activity of the ureide enzymes under stress would be beneficial. Moreover, studying the activities of antioxidant enzymes in *aln-3* mutant plants and/or in response to exogenous application of allantoin in wild-type plants might be informative. Additionally, addressing the posttranslational regulation of proteins involved in this process may provide better insight into the physiological functions of these proteins or clues to the gene regulatory mechanisms. Finally, identification and study of ureide transporters was not included in this research, but finding these transporters that contribute to the transporting of ureides inside the cell would provide instructive information to further ureide research.

Finally, in order to fully utilize this knowledge more investigations should be conducted since there are still many questions remaining to be answered. For example, are the other compounds and genes in the ureide pathway only involved in recycling nitrogen or are they also involved in stress or ABA response? An interesting report in late 2015 suggested that ureidoglycolate amidohydrolase (UAH), the last enzyme in the ureide metabolism pathway, is involved in the regulation of low temperature response but not ABA in rice (Li *et al.*, 2015). In contrast to a dramatic increase in the expression of *UAH* in response to low temperature, ABA treatment had no effect on *UAH* transcript at any tested time point (Li *et al.*, 2015). Therefore, more studies will help us to understand the regulation and function of ureide metabolism genes that may contribute to plant stress responses.

As my research and recent reports indicate, the use of allantoin (and other potential ureides) in order to enhance plant tolerance, or as a marker for stress, under both abiotic and biotic stresses is highly promising. It has been suggested that allantoin can be a potential metabolic marker of drought (Degenkolbe *et al.*, 2013) and salt stress (Nam *et al.*, 2015) response in rice, since it probably is a conserved metabolic marker in most of the rice genotypes. Further research in the direction to answer outstanding remaining questions would particularly assist the establishment of beneficial applications in the future.

## 5. APPENDIX A. Cloning

### **Generation of tools to study the expression of a number of ureide metabolism coding genes and their overexpression**

#### **A1. INTRODUCTION**

Development of modified insertion element systems such as fusions of promoterless reporter genes with the promoter sequence of genes of interest provides the way to analyze the gene expression of the gene of interest. These systems have been designed to use the reporter genes to monitor the expression of individual genes by identification of the location and time of expression of the tagged promoter (Springer, 2000). In my research, the promoter-reporter constructs of a number of Arabidopsis ureide metabolism genes were constructed. Ureide gene promoters were inserted in a vector to drive expression of  $\beta$ -glucuronidase (GUS) and green fluorescent protein (GFP) in plants. These constructs will be used in future experiments to address questions regarding when or where (what cells) genes involved in ureide metabolism are expressed following stress treatment.

In 1987, the GUS encoding gene,  $\beta$ -D-glucuronidase in *Escherchia coli* was introduced as a new tool to assay the activity of genes in transgenic plants (Jefferson *et al.*, 1987). The GUS gene can be detected easily by histochemical, spectrophotometrical or fluorimetical tools based on type of the substrate used for the assay (Jefferson *et al.*, 1986). The *GUS* reporter gene can act as a tag for identification of the expression pattern of a gene in a cell under different developmental stages or abiotic stress conditions (Jeon *et al.*, 2000). Green fluorescent protein (GFP) derived from coelenterates such as pacific jellyfish (*Aequorea victoria*) (Chalfie *et al.*, 1994) is a spontaneously fluorescent protein that can be used as a protein tag (Yang *et al.*, 1996). *GFP* was first cloned by Prasher *et al.* (1992) and used as a marker to study gene expression by Chalfie *et al.* (1994). The fluorescent protein has 238 amino acids with the ability to absorb blue light (395 nm) and

emission of green light (509 nm). Fusion of GFP coding sequence to host genes with known or unknown functions has enabled scientists to localize the expressed genes and their encoding proteins movement through different cell compartments (Hanson and Kohler, 2001).

In the present research, the pCAMBIA1303 vector has been used as cloning vector for plant transformation. pCAMBIA vectors were derived from pZAP vectors by Hajdukiewicz *et al.* (1994) and some of their advantages are: high copy number in *E.coli*, high stability in *Agrobacterium*, chloramphenicol or kanamycin selection for bacteria and hygromycin B or kanamycin selection for plants. pCAMBIA1303 has both GFP and GUS reporter genes available as *gusA-mgfp5-His6* fusion (<http://www.cambia.org>).

In addition to promoter-reporter constructs, I have also constructed a number of ureide gene overexpression constructs. The questions to answer were: does overexpression of ureide genes change plant abiotic stress response or stress tolerance? Overexpression studies were initiated after the development of techniques for yeast transformation (Beggs 1978; Hinnen *et al.*, 1978) and genomic library construction (Nasmyth and Reed, 1980; Carlson and Botstein, 1982). However, overexpression research has not been restricted to yeast and have been successfully used in a number of organisms such as *Xenopus*, *Drosophila* and *Arabidopsis* (Prelich, 2012). Studying the overexpression of genes has contributed much to our understanding and knowledge of molecular biology and provides a research tool with distinct advantages such as overexpression of wild-type protein in mutant background, phenotype studies in epistasis tests and identification of drug targets (Prelich, 2012). Stress response genes or their regulator genes can be transferred to and expressed in *Arabidopsis* using *Agrobacterium* mediated transformation which can lead to biochemical and physiological changes in growth and development of plants under stress. Overexpression has been widely used to study plant transcriptional regulation, osmoprotectants and stress signaling (Ahmad and Rasool, 2014).

In general, I amplified the coding sequence of a number of ureide genes using PCR and inserted them into pCAMBIA 1303, with expression driven by CaMV 35S, a strong promoter of the Cauliflower Mosaic Virus (CaMV). Cauliflower Mosaic Virus affects many plants in the family Brassicaceae (Schoelz and Bourque, 1999). The genome of CaMV is a double stand circular DNA of 8 kbp with two main RNA transcripts of 19S and 35S (Ho *et al.*, 1999). The CaMV 35S promoter has 350 bp upstream of the 35S transcript and about 250 bp which overlaps with the end of the open reading frame at the previous gene. The promoter has three domains; TATA box and two



major enhancers. The 35S promoter is an efficient constitutive promoter and its functionality has been confirmed in many organisms other than plants such as bacteria and animals (Hull *et al.*, 2000). From the CaMV 35S promoter, different hybrids or combination promoters have been constructed to drive transgene expression in transgenic plants, such as hybrids of 19S and 35S enhancers or double 35S promoters (Ho *et al.*, 1999). pCAMBIA vectors have a double-enhancer CaMV35S promoter ([www.cambia.org](http://www.cambia.org)).

The promoter::reporter tools that were developed in this part will be used to localize ureide metabolism enzymes under controlled environmental conditions using the fusion of *GFP* or *GUS* under the control of promoters of ureide metabolism genes. The overexpression ureide tools will be used to study the effect of overexpression of ureide metabolism enzymes on plant abiotic stress response using a fusion of ureide genes to the strong CaMV 35S promoter.

## **A2. MATERIALS AND METHODS**

### **A2.1. Ureide promoter region prediction**

The promoter of *XDHI*, *UO*, *ALN* and *AAH* in this research was estimated using information from prediction software, mainly TSSP program (<http://www.softberry.com>) and Promoter Scan (<http://www-bimas.cit.nih.gov/molbio/proscan/>). Transcription element locations were also predicted by software PLACE (Higo *et al.*, 1999) (<http://www.dna.affrc.go.jp/database/>).

Promoter Scan is a computer program which has been developed and publicized in 1995 by Dr. Dan Prestridge (Molecular Biology Computing Center, University of Minnesota). This program uses a recognition algorithm for the majority of eukaryotic RNA polymerase II promoters (70% correct promoter recognition) with a small false positive rate (~1/5565 bases in non-promoter sequence set) (Prestridge, 1995). In simple terms, Promoter Scan reports the estimated promoter regions such as TATA box or transcriptional start site position (TSS) (Prestridge, 1995). TSSP is another computer program that works with a PromH algorithm with an ability to localize both promoter and TSS which makes it a more sensitive and more specific algorithm than previous ones. PromH can localize transcription starting sites (TSS), TATA boxes, known regulatory elements around the TSS and conserved regulatory motifs in orthologous gene pairs (Solovyev

and Shahmuradov, 2003). In the present research, in order to amplify the genes promoter, I used ‘core promoter’ upstream the start codon (several hundred nucleotides around the transcription start site which is believed to direct RNA polymerase II to a right position) and ‘distal promoter’ region (up to a few thousands of nucleotides upstream transcription start site) to design primers.

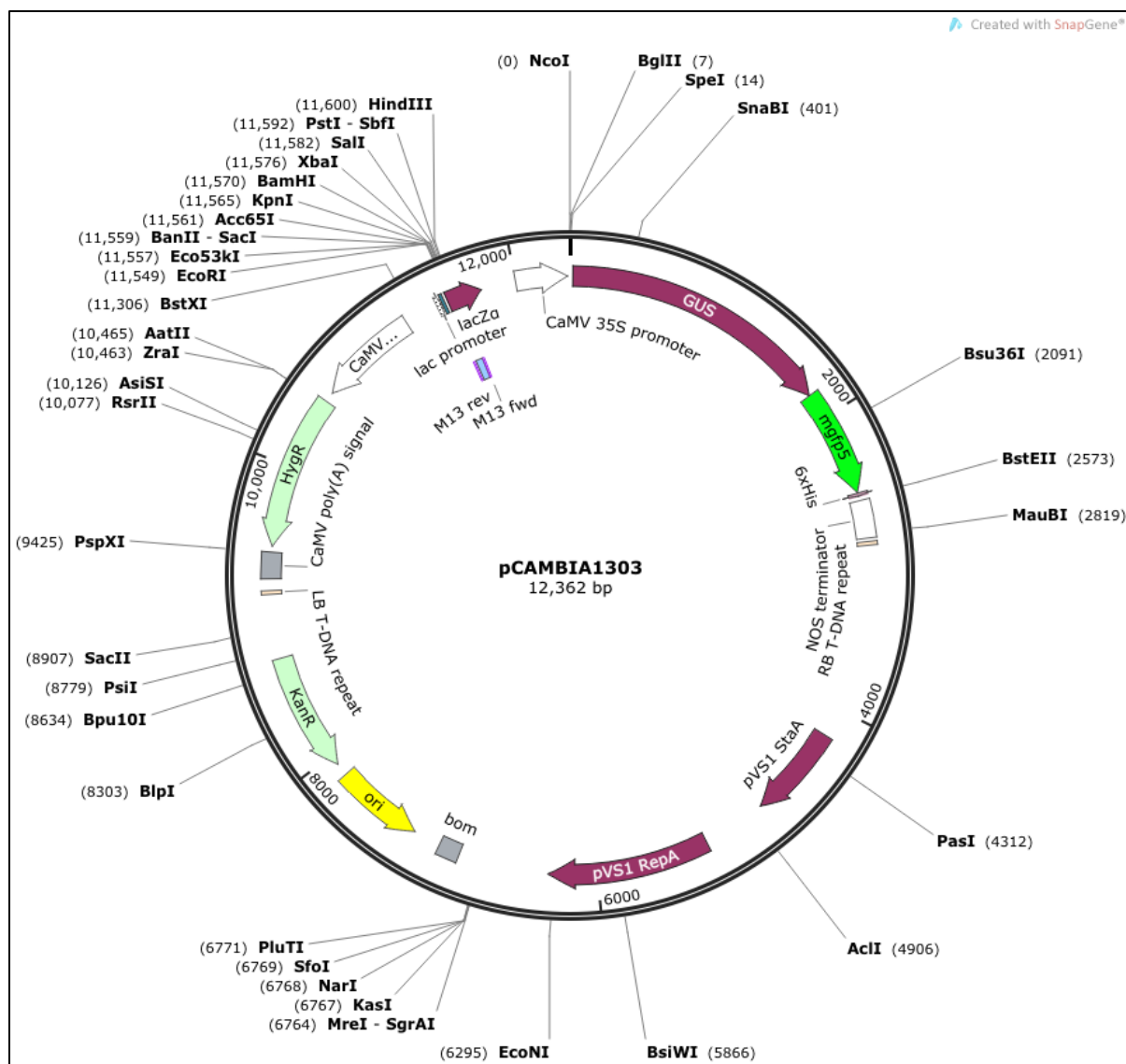
### **A2.1. Construction materials and primers**

In the present research, pCAMBIA1303 has been used as the transformation vector (Figure A1). For initial cloning and sequencing of PCR products the pCR<sup>TM</sup>-Blunt II- TOPO plasmid vector (3519 bp) from the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, CA, USA) was used. Electro-competent *E. coli* and *Agrobacterium tumefaciens* were prepared according to the procedures described by Sambrook and Russell (2001).

Primers that designed to amplify a promoter or coding sequence of ureide metabolism genes are listed in table A1. To choose restriction enzymes which do not cut the insert sequence, NEBcutter V2.0 (New England BioLabs; [www.neb.com/NEBcutter2](http://www.neb.com/NEBcutter2)) was used. No restriction enzyme sites were used for cloning the *UO* promoter since all the potential restriction enzymes would also cut the *UO* promoter (this promoter will be cloned using a blunt end cloning method). Where restriction sites were included in PCR primers, the overhang sequence of CACACA was added to the 5’ of each primer. The overhang sequence improves direct digestion of PCR products since it moves the added restriction enzyme site away from end of the PCR product.

### **A2.3. Standardization of PCR Conditions**

An Eppendorf thermal cycler (Eppendorf MasterCycle Gradient, Germany) was used for all PCRs. *Pfu* DNA polymerase, PCR buffer + MgCl<sub>2</sub> (Fermentas; ThermoFisher Scientific Waltham, MA, USA) and deoxyribonucleotide mix (BioShop, Burlington, ON, Canada) were used as the PCR reagents. The final concentration of PCR reagents in the PCR was: 1X PCR buffer, 0.3 µM of each primer, 0.05 units/µL *Pfu* DNA polymerase and 200 µM deoxyribonucleotide mix. PCR conditions were standardized to amplify the promoters or coding sequences using the specific primers with overhang. Table A2 shows the optimized PCR conditions for each target.



**Figure A1. pCambia1303 vector structure.** pCambia1303 contains both GFP and GUS reporter genes as a *gusA-mgfp5*-His6 fusion and CaMV 35S promoter. The image is taken from Cambia website (<http://www.cambia.org>).

**Table A1. List of primers used to amplify the promoter or coding sequences of ureide genes.** Red nucleotides in the primer sequence represent restriction enzyme sites and the gray nucleotides are extra overhangs. Sequences of the primers are presented as 5' to 3'.

Primer	Gene	Product length
CACACA <b>GAATTC</b> GGACCCGGAAGCTACAAAAT	Xanthine dehydrogenase 1 ( <i>XDHI</i> ) (at4g34890) Promoter	1084- Forward
CACACA <b>CCATGG</b> AAATTGAGAGAAAACCTCGATTGG	Xanthine dehydrogenase 1 ( <i>XDHI</i> ) (at4g34890) Promoter	1084- Reverse
CACACA <b>ACTAGT</b> ATGGGTTCAGTAAAAAGGACG	Xanthine dehydrogenase 1 ( <i>XDHI</i> ) (at4g34890) Coding sequence	4119- Forward
CACACA <b>GGTGACCT</b> CGATCTGTTTCCCTCTGA	Xanthine dehydrogenase 1 ( <i>XDHI</i> ) (at4g34890) Coding sequence	4119- Reverse
CACACA <b>GAATTC</b> GAGTGTATGTGTTAGCCACGGTA	Allantoinase ( <i>ALN</i> ) (At4g04955) Promoter	1514- Forward
CACACA <b>CCATGG</b> GATCTCTTCTGCGATTATTTGGA	Allantoinase ( <i>ALN</i> ) (At4g04955) Promoter	1514- Reverse
CACACA <b>CCATGGT</b> GAGAGAACTTTGCTTCAATGG	Allantoinase ( <i>ALN</i> ) (At4g04955) Coding sequence	1603- Forward
CACACA <b>GGTCACCT</b> TGCAACATGAGAGCAACTCTT	Allantoinase ( <i>ALN</i> ) (At4g04955) Coding sequence	1603- Reverse
CACACA <b>GAATTC</b> TGCTTAAAGAGCATGGATCAGA	Allantoate Amidohydrolase ( <i>AAH</i> ) (at4g20070) Promoter	1133- Forward
CACACA <b>ACTAGT</b> GCTGCCGATTAAAGAGGTTG	Allantoate Amidohydrolase ( <i>AAH</i> ) (at4g20070) Promoter	1133- Reverse

CACACA <b>ACTAGT</b> ATGGCGGTTCTCATCCTTCTTCTT	Allantoate Amidohydrolase ( <i>AAH</i> ) (at4g20070) Coding sequence	1694- Forward
CACACA <b>GGTCACCT</b> GTCAGTTCCAAAGAGTACACAGA	Allantoate Amidohydrolase ( <i>AAH</i> ) (at4g20070) Coding sequence	1694- Reverse
TATAAAGCTCGGGGGCAAG	Uricase ( <i>UO</i> ) (At2g26230) Promoter	1692- Forward
TCAAACCAAATTGATACATAGAAACA	Uricase ( <i>UO</i> ) (At2g26230) Promoter	1692- Reverse
CACACA <b>CCATGG</b> CACAAGAAGCCGATGGGATC	Uricase ( <i>UO</i> ) (At2g26230) Coding sequence	996- Forward
CACACA <b>GGTCACC</b> AACGCCAAAAGTTGCAGAAC	Uricase ( <i>UO</i> ) (At2g26230) Coding sequence	996- Reverse

**Table A2. PCR conditions used for amplification of each sequence.**

*XDHI*: xanthine dehydrogenase 1; *UO*: uricase; *ALN*: allantoinase; *AAH*: Allantoate Amidohydrolase

Gene	PCR condition
<i>XDHI</i> -Coding sequence	Initial denaturation 95 °C 3 min, 32 cycles of 95 °C 30 sec, 60 °C 40 sec, 72 °C 1 min, final extension 72 °C 4 min
<i>UO</i> -Coding sequence	Initial denaturation 96 °C 3 min, 34 cycles of 94 °C 30 sec, 64 °C 55 sec, 72 °C 90 sec, final extension 72 °C 4 min
<i>ALN</i> -Coding sequence	Initial denaturation 96 °C 3 min, 32 cycles of 95 °C 30 sec, 60 °C 40 sec, 72 °C 1 min, final extension 72 °C 4 min
<i>AAH</i> -Coding sequence	Initial denaturation 95 °C 2:30 min, 30 cycles of 95 °C 30 sec, 58 °C 30 sec, 72 °C 40 sec, final extension 72 °C 3 min
<i>XDHI</i> -Promoter	Initial denaturation 95 °C 3 min, 30 cycles of 95 °C 30 sec, 62 °C 60 sec, 72 °C 60 sec, final extension 72 °C 4 min
<i>UO</i> -Promoter	Initial denaturation 95 °C 3 min, 33 cycles of 95 °C 30 sec, 60 °C 45 sec, 72 °C 50 sec, final extension 72 °C 4 min
<i>ALN</i> -Promoter	Initial denaturation 95 °C 2:30 min, 30 cycles of 95 °C 30 sec, 62 °C 30 sec, 72 °C 30 sec, final extension 72 °C 3 min
<i>AAH</i> -Promoter	Initial denaturation 95 °C 2:30 min, 30 cycles of 95 °C 30 sec, 58 °C 30 sec, 72 °C 40 sec, final extension 72 °C 3 min

#### **A2.4. Cloning of PCR products into the TOPO vector**

The Zero Blunt TOPO PCR Cloning Kit containing the Pcr<sup>TM</sup>-Blunt II- TOPO plasmid vector was used for the initial cloning of PCR products. The TOPO cloning reaction was done using fresh PCR product, sterile water, salt solution (provided in the kit) and TOPO vector (provided in the kit) according to the protocol recommended by the manufacture. Once the TOPO cloning reaction was done, competent *E. coli* was transformed by electroporation. To perform electroporation, the TOPO cloning reactions were added to the competent *E. coli* in 2 mm electroporation cuvettes (VWR International, Mississauga, ON, Canada). For electroporation, a Bio-Rad electroporation system (Gene Pulser II model) with setting at 1.8 kV and a capacitance of 25  $\mu$ F (Sambrook and Russell, 2001) was used. After electroporation, the solution was transferred to 1 mL LB (Luria-Bertani) liquid medium containing 0.5% w/v bacto-yeast extract (BioShop), 1% tryptone (Becton, Dickinson and Company; USA), 1% NaCl at pH 7.0 supplied with 50 mg L<sup>-1</sup> Kanamycin (Calbiochem; VWR). Electroporated samples were incubated at 37°C in an incubator shaker (1575 model; VWR) for 1 hour and then plated on LB agar media containing kanamycin. Positive colonies were apparent after 24 hours. Plasmids from positive colonies were isolated using the HiYield Plasmid Mini Kit (RBC Bioscience, BioAspect Inc, ON, Canada). To confirm the presence of the desired DNA fragments in the cloning vector, plasmids were digested with restriction enzyme EcoRI (NEB, New England Biolabs, Ipswich, MA, USA) and separated on a 1.2 % agarose gel. Positive plasmids were sequenced using Sanger sequencing method (NRC, Saskatoon) by M13 forward (5'-GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers and sequence alignment was done using Align and Blast algorithm available at NCBI (<http://www.ncbi.nlm.nih.gov>).

#### **A2.5. Restriction endonuclease digestion**

Restriction endonuclease enzymes (New England Biolabs) were used in double restriction digestion reactions. The digested sample was analysed by agarose gel electrophoresis and the desired fragments were eluted from the gel by E.Z.N.A gel extraction Kit (OMEGA Bio-Tek Inc, VWR) according to the manufacture's recommended protocol. The DNA insertions cloned into the pCAMBIA vector are listed in table A3.

**Table A3. Restriction enzymes used for double-digestion of *XDHI* promoter and *UO* coding sequence.**

Gene	Restriction Enzymes	Supplied buffer	Product size (bp)
Xanthine dehydrogenase 1 ( <i>XDHI</i> ) (at4g34890) Promoter	EcoRI (G <sup>^</sup> AATTC) NcoI (C <sup>^</sup> CATGG)	Tango buffer (New England Biolabs)	1084
Uricase ( <i>UO</i> ) (At2g26230) Coding sequence	NcoI (C <sup>^</sup> CATGG) BstEII (G <sup>^</sup> GTNACC)	NEB buffer 3.1	996



## **A2.6. Ligation**

For ligation of genes in the pCAMBIA vector, T4 DNA ligase (New England Biolabs) was used. For ligation reactions, a 1:3 ratio of vector to insert DNA in 20 µl sterile ddH<sub>2</sub>O was used. The ligation reaction was completed at 23°C for 1 h.

## **A2.7. Recombinant pCAMBIA 1303 cloning**

*E. coli*, strain DH5α was transformed with the recombinant vectors by electroporation at 1.8 kV and a capacitance of 25 µF (Sambrook and Russell, 2001). pCAMBIA 1303 lacking any insert was also introduced into the bacteria to provide a control vector.

## **A2.8. Agrobacterium transformation**

Binary vector construct: pCAMBIA+ *UO* coding sequence, pCAMBIA+ *XDHI* promoter and pCAMBIA (without insertion) were transferred into competent *A. tumefaciens*, strain GV1301, by electroporation at 2.5 kV and a capacitance of 25µF. After electroporation, the bacterial suspensions are transferred to 1 ml LB media and incubated in the shaker at 28° C for 1 h. The incubated suspension was transferred to LB agar plates containing 50 mg L<sup>-1</sup> kanamycin and incubated at 28° C for 48 h. After 48 h, colonies were screened by colony PCR using the specific primers for each gene.

## **A2.9. Plant transformation**

*A. thaliana* plants, ecotype Colombia (Col-0) were transformed using the floral dip method as described by Zhang *et al* (2006). Arabidopsis seeds were germinated on MS media and allowed to grow for 10 days. About 8-10 seedlings were transferred to each individual 10 cm (350 mL volume) pot containing soil. Pots were placed in long-day conditions (16/8 h light/dark cycle, 22 °C) for 4 to 5 weeks until the plants contained approximately 20–25 inflorescences and probably some maturing siliques. Mature siliques were clipped off before transformation. Agrobacterium was prepared by transferring a single colony of Agrobacterium into 5 mL LB medium containing kanamycin (50 mg L<sup>-1</sup>). The culture was incubated at 28° C for 48 h and used as a feeder culture to inoculate 500 mL liquid LB and grow at 28° C for 1 additional day. The bacterial cells were collected at room temperature by centrifugation at 4,000g for 10 min. The collected bacteria were suspend in 500 mL of 5% (wt/v) sucrose solution. 0.02% (v/v) (100 µL per 500 mL of solution)

Silwet L-77 (Phytotechnology Labs, KS, USA) was added to the solution immediately before dipping. The plants were inverted in the *Agrobacterium* suspension, with aerial parts of the plants dipped in suspension for 10 s. Plants were allowed to cover under a plastic cover overnight and were shifted back to the chamber for growth and maturation of siliques. T<sub>1</sub> seeds from mature siliques of dried plants were collected. T<sub>1</sub> seeds were planted and screened on MS medium containing 25 mg/L hygromycin (ThermoFisher Scientific). After 1 week, potential transformants were transferred to a fresh selection plate with hygromycin for an additional 10 days to ensure they were true transgenic lines.

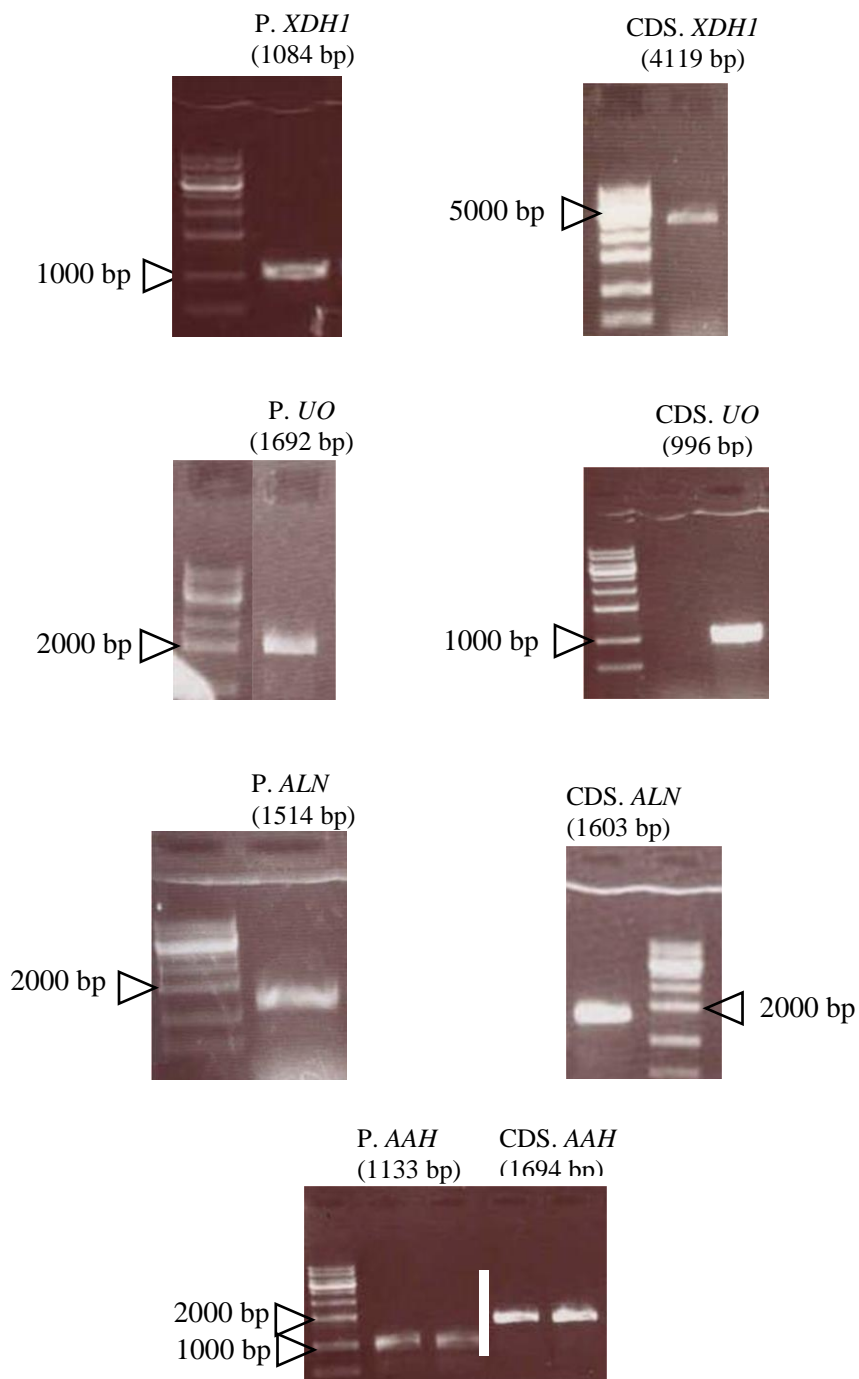
### **A3. RESULTS**

#### **A3.1. Amplification of promoter and CDS sequences**

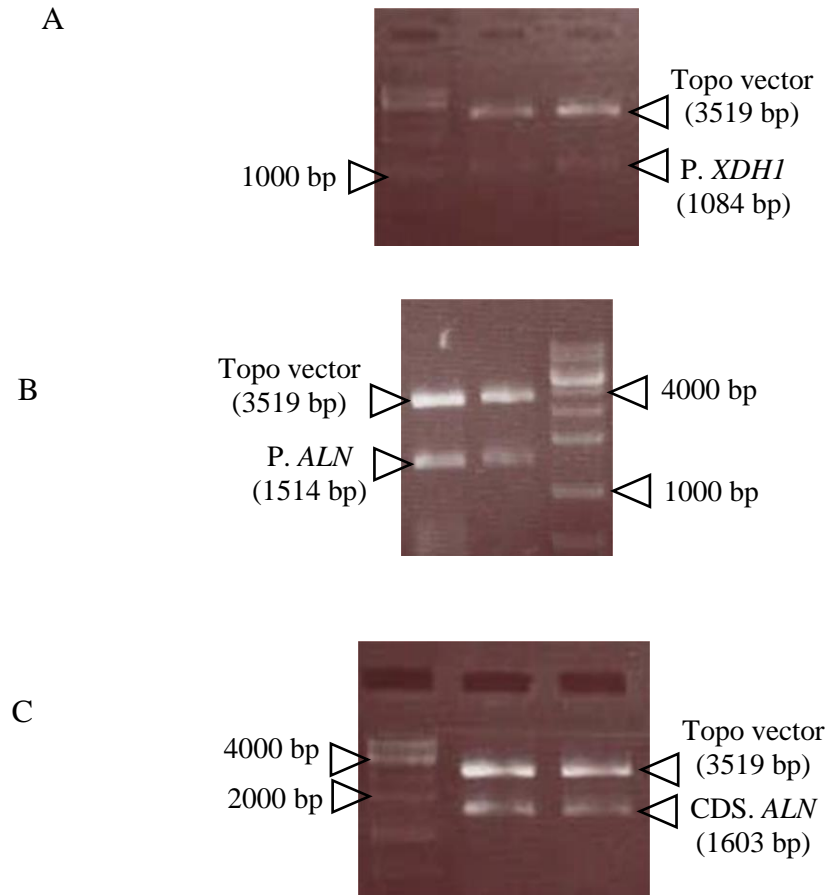
PCR conditions were standardized to amplify the DNA fragment for four promoter and four CDS regions as shown on Figure A2.

#### **A3.2. Cloning of PCR product into TOPO vector**

Following PCR amplification, a number of PCR products were cloned into Pcr<sup>TM</sup>-Blunt II-TOPO plasmid vector. Plasmid was extracted from positive colonies and digested by restriction enzyme EcoRI to confirm the insertion (Figure A3). Cloned fragments were confirmed by sequencing and analysis with BLAST before sub-cloning into the plant transformation vector, pCAMBIA 1303.



**Figure A2. PCR amplification products of promoter and coding sequence of four ureide metabolism genes using primers with restriction enzyme sites and extra overhang nucleotides.** *XDHI*: xanthine dehydrogenase 1; *UO*: uricase; *ALN*: allantoinase; *AAH*: Allantoate Amidohydrolase. P: promoter; CDS: coding sequence.



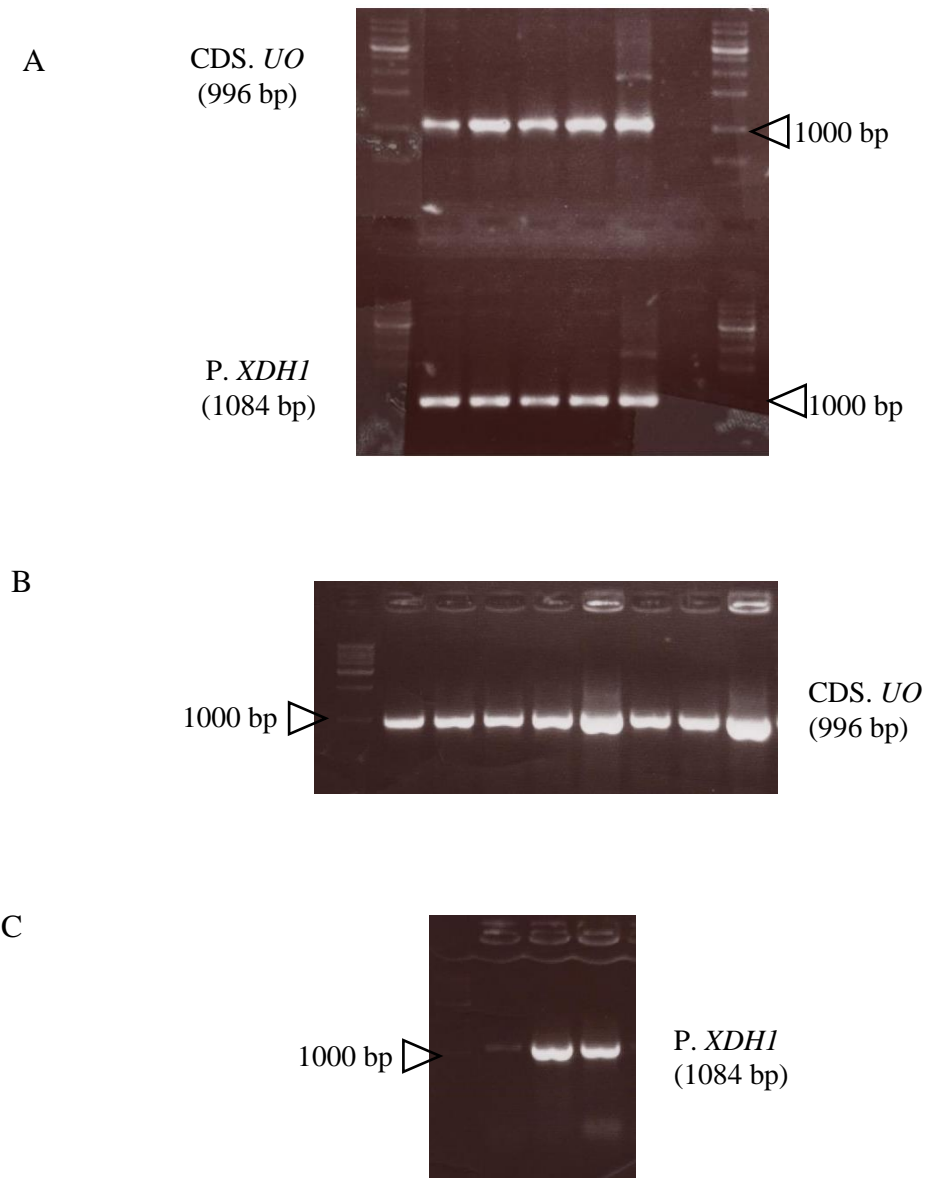
**Figure A3. EcoRI restriction enzyme analysis of a number of ureide biosynthesis gene insert in TOPO plasmid vector.** (A) Restriction enzyme digestion of *XDH1* promoter (B) Restriction enzyme digestion of *ALN* promoter (C) Restriction enzyme digestion of *ALN* coding sequence. All digestions are done with EcoRI enzyme. *XDH1*: xanthine dehydrogenase 1; *ALN*: allantoinase. P: promoter; CDS: coding sequence

### **A3.3. Bacterial transformation**

After sequencing, the *XDHI* promoter and *UO* coding sequences were sub-cloned into the pCAMBIA 1303 vector. Recombinant pCAMBIA1303 vectors were first introduced into *E. coli* and then into *A. tumefaciens*. A pCAMBIA1303 vector without insertion was also used to transform bacteria to provide a control vector. To confirm the transformation of *E.coli* and *A. tumefaciens*, positive clones were used for PCR amplification of the desired gene sequences (Figure A4).

### **A3.4. Agrobacterium-mediated Arabidopsis transformation**

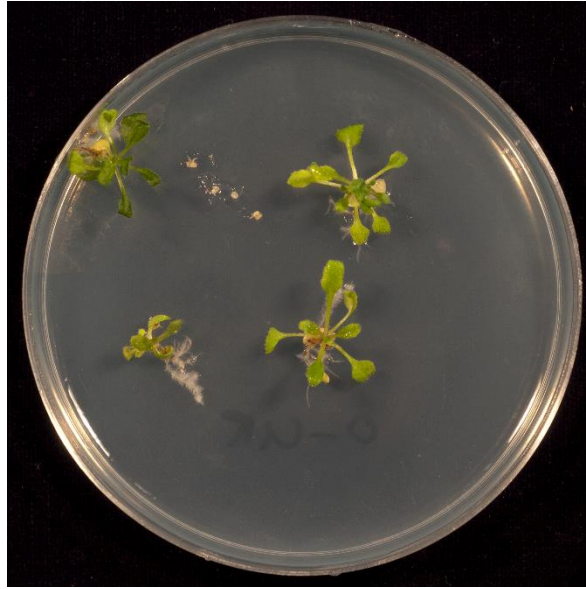
T<sub>1</sub> seeds generated from Arabidopsis plants after floral dip transformation were screened on MS plates containing hygromycin (25 mg L<sup>-1</sup>) (Figure A5). On hygromycin selection plates, only transformed seedlings develop roots that penetrate the media and produce true leaves (Zhang *et al.*, 2006). After 1 week, potential transformants were transferred to a fresh selection plate for an additional 10 days to ensure they were true transgenic seedlings (Figure A6). Seedlings were transferred into soil and were allowed to grow and set seed. T<sub>2</sub> seeds were collected and stored for future experiments.



**Figure A4. Colony-PCR results of positive *E. coli* and *A. tumefaciens* colonies.** (A) Colony-PCR of positive *E. coli* colonies for *P. XDHI* and CDS.*UO*. (B) Colony-PCR of positive *A. tumefaciens* colonies for CDS. *UO* colonies. (C) Colony-PCR of positive *A. tumefaciens* colonies for *P. XDHI*. *XDHI*: xanthine dehydrogenase 1; *UO*: uricase. P: promoter; CDS: coding sequence



**Figure A5. Identification of T<sub>1</sub> transformant seedlings on MS with 25 mg L<sup>-1</sup> hygromycin selection plates.** When seeds are planted on hygromycin selection media, only true transformants (shown in red circles) develop roots inside the media and produce true leaves.



**Figure A6. Potential T<sub>1</sub> transformant seedlings after 10 days growth on fresh hygromycin selection plate.** Potential T<sub>1</sub> transformant seedlings were transferred to a fresh selection media and were allowed to grow for an additional 10 days to ensure that they were true transgenic lines before transferring them to pots containing soil. The picture shows transgenic *UO* overexpression plants. *UO*: uricase



#### A4. Discussion

Application of reverse genetic approaches such as overexpression, knockout mutants and reporter genes has remarkably helped scientists to study the function of Arabidopsis stress-responsive genes (Visscher *et al.*, 2015). Overall, I have designed primers to amplify the potential promoter region as well as the coding sequence of the four Arabidopsis ureide metabolism genes *XDH1*, *UO*, *ALN* and *AAH*. PCR products from each amplification was initially introduced into the pCR<sup>TM</sup>-Blunt II- TOPO plasmid vector and confirmed by sequencing. After confirmation of no sequence mismatch in the PCR products, the *XDH1* promoter and *UO* coding sequences were transferred to the pCAMBIA 1303 vector and then to Arabidopsis plants via Agrobacterium transformation system. Although all PCR products from this research will eventually be used to transform Arabidopsis, I have selected to start the cloning procedures with the *XDH1* promoter and *UO* coding sequences.

Phenotypic characterization of Arabidopsis *XDH*-knockdown mutants provided the first evidence on function of ureide metabolism in plant physiological processes (Nakagawa *et al.*, 2007) and abiotic stress response (Watanabe *et al.*, 2010). It has been suggested that XDH is involved in some of the main physiological aspects of plant life such as hypersensitive cell death (Montalbini, 1995), drought stress response (Yesbergerova *et al.*, 2005; Watanabe *et al.*, 2010) and senescence (Pastori and Del Rio, 1997; Brychkova *et al.*, 2008). It is still undetermined whether XDH activity contributes to the regulation of ROS levels in plant cells in response to abiotic or biotic stresses (Nakagawa *et al.*, 2007). Thus, for example, *XDH1* promoter::reporter plants can be used to study the expression of *XDH1* as well as localization of ROS at the same time under stress conditions.

The first plant *uo* mutant and its phenotype was reported by Hauck *et al.* (2014) in Arabidopsis. Increased level of uric acid in the *Atuo* mutant resulted in a lower germination rate and severe deficiency in seedling establishment under non-stress conditions. Moreover, as presented in the current thesis, higher expression of *UO* was found under various abiotic stresses in Col-0 plants. Simultaneously, accumulation of allantoin was determined in Col-0 plants under stress conditions. All of these observations suggest that studying *UO* overexpressed plants under abiotic stress can be noteworthy.

Cloning of ureide metabolism genes will help us to investigate their expression using promoter::reporter lines or their response to abiotic stress after upregulation using overexpression lines under adverse environmental conditions. These constructs can subsequently be used to transform *Arabidopsis* for reverse genetic studies. Therefore, transgenic plants or the constructs achieved in this research will help us to study the function of these genes in response to high salinity, drought or other abiotic stresses in *Arabidopsis*.

## 6. APPENDIX B. Supplementary tables

**Table B1. T-DNA insertion lines ordered from ABRC listed in the order of the ureide metabolic pathway.**

<i>A. thaliana</i> Gene	T-DNA insertion line code	T-DNA insertion location
<i>XDH1</i> (At4g34890)	SALK_ 148364	On 10 <sup>th</sup> interon from 13
<i>XDH1</i> (At4g34890)	SALK_ 148366	On 10 <sup>th</sup> interon from 13
<i>XDH2</i> (At4g34900)	SALK_ 143041	On 4 <sup>th</sup> exon from 14
<i>XDH2</i> (At4g34900)	SALK_ 015081	On 4 <sup>th</sup> exon from 14
<i>UO</i> (At2g26230)	SALK_ 131436	On 1 <sup>st</sup> intron from 8
<i>UO</i> (At2g26230)	SALK_ 096471	5' UTR
<i>UO</i> (At2g26230)	SAIL_ 267b_D04	On 1 <sup>st</sup> exon from 8
<i>ALN</i> (At4g04955)	SALK_ 013986	On 12 <sup>th</sup> exon from 15
<i>ALN</i> (At4g04955)	SALK_ 142607	On 9 <sup>th</sup> intron from 14
<i>ALN</i> (At4g04955)	SALK_ 000282	On 12 <sup>th</sup> exon from 15
<i>ALN</i> (At4g04955)	SAIL_ 810_E12	On 2 <sup>nd</sup> exon from 15
<i>AAH</i> (At4g20070)	SALK_ 112631	On 10 <sup>th</sup> interon from 11
<i>UGLYAH</i> (At4g17050)	SALK_ 054835	On 11 <sup>th</sup> exon from 13
<i>UGLYAH</i> (At4g17050)	SALK_ 071989	On 1 <sup>st</sup> exon from 13
<i>UGLYAH</i> (At4g17050)	SALK_ 072119	Promoter
<i>UAH</i> (At5g43600)	SALK_ 108394	Promoter
<i>UAH</i> (At5g43600)	SALK_ 024998	On 2 <sup>nd</sup> intron from 11
<i>UAH</i> (At5g43600)	SAIL_ 338_C08	On 10 <sup>th</sup> exon from 12

**Table B2. Sequence of primers designed with the T-DNA primer design program and used to screen T-DNA insertion lines.** Sequences of the primers presented as 5' to 3' and genes are listed in order of the ureide metabolic pathway.

<b>Forward primer</b>	<b>Reverse primer</b>	<b>T-DNA mutant line</b>	<b><i>A. thaliana</i> Gene</b>
ATTCAAAGAATCGCAGCTGAG	GCGTTCCCTTTTCCAGATATC	SALK_148366.55.75.x	<i>XDH1</i> (At4g34890)
ATTCAAAGAATCGCAGCTGAG	GCGTTCCCTTTTCCAGATATC	SALK_148364.54.75.x	<i>XDH1</i> (At4g34890)
ATTGGCATAACGACAGAGACAAC	CTGCAAGACGCACTTAAGGTC	SALK_143041.54.75.x	<i>XDH2</i> (At4g34900)
ATTGGCATAACGACAGAGACAAC	GATTAAGGAAGGTGCTCCTGG	SALK_015081.51.10.x	<i>XDH2</i> (At4g34900)
GAGCTAGTCCTCCAATACCGG	TGATACAAGGCGAAATCGATC	SALK_131436.46.30.x	<i>UO</i> (At2g26230)
GAGCTAGTCCTCCAATACCGG	GGTATTTACCGAAAATGGCC	SALK_096471.54.00.x	<i>UO</i> (At2g26230)
AGATGCATTAACCTCTGTGGC	TGAGAGTTGGAAGAGTTTGGC	SAIL_267b_D04/CS812424	<i>UO</i> (At2g26230)
CACGCAGAGATCGAGAGAGAC	TACATCAAATTCGGCTTCAGG	SALK_000282.55.25.x	<i>ALN</i> (At4g04955)
CACGCAGAGATCGAGAGAGAC	TACATCAAATTCGGCTTCAGG	SALK_013986.54.05.x	<i>ALN</i> (At4g04955)
GCTGAATAAAGCACGTAAGCG	AATATCTCACCACAACCGTCG	SAIL_810_E12 / CS836203	<i>ALN</i> (At4g04955)
CGCTTACGTGCTTTATTGAGC	CACTGTCTCCTTTGCCTTTTG	SALK_142607.50.20.x	<i>ALN</i> (At4g04955)
CATGCAATGGACAATACCATG	TGAAAAGTGTTCGAAAAACC	SALK_112631.51.60.x	<i>AAH</i> (At4g20070)
AGTGCTTAGAGTGGACATGGC	ATTGTTGGCTCCACAGACAAG	SALK_054835.54.65.x	<i>UGLYAH</i> (At4g17050)
ATTTCCCTCAATTCACCACC	TTCGTGGCTTTCCAATAAATG	SALK_071989.54.50.x	<i>UGLYAH</i> (At4g17050)
ATTTCCCTCAATTCACCACC	TTCGTGGCTTTCCAATAAATG	SALK_072119.42.00.x	<i>UGLYAH</i> (At4g17050)
GAGTGGGAGATTGAAACGAC	GAGCCGAGACATCTTTGTCAG	SALK_108394.47.50.x	<i>UAH</i> (At5g43600)
TTCCCTGTCACAATCTAAGAGC	TGAGCTCTTGAGGAAGCTGAG	SAIL_338_C08/ CS815741	<i>UAH</i> (At5g43600)
CTTCTGCGTATCCTGCTGATC	TCTGCTCTATTGCGCTTCTTC	SALK_024998.56.00.x	<i>UAH</i> (At5g43600)

**Table B3. List of primers used to detect the effect of T-DNA insertion on the expression of the impaired gene by RT-PCR.** Sequences of the primers presented as 5' to 3' and genes are listed in order of the ureide metabolic pathway.

<i>A. thaliana</i> Gene	Abbreviation	Forward Primer	Reverse Primer	Product size (bp)	Genome size (bp)	Transcript size (bp)
AT4G34890	<i>XDH1</i>	CGCGGATACACATGAAAATG	TTCCAGAGCTGATGCAATG	904	6485	4323
AT4G34900	<i>XDH2</i>	GTGGCTTTGGTGGTAAGGAA	CGTCTAAAACTGCAGCACCA	891	5773	4216
AT2G26230	<i>UO</i>	GAAAGCCAAGGAATGTGGAG	TGAAGGGTTTCCTTCGTTG	662	2621	1354
AT4G04955	<i>ALN</i>	GAACTAAGGCTGCTGCTGCT	TTCAGGTTCCACACAACAA	977	3247	1809
AT4G20070	<i>AAH</i>	AAGGGACGAAGCTGTAGCAA	TAGCACTTGGCCATGTTGAG	827	3242	1838
AT4G17050	<i>UGLYAH</i>	GCCCTTTACCTGACTGGACA	AGTCTTTCCGAGTGCAGCAT	611	3021	1166
AT5G43600	<i>UAH</i>	ATCTGCCTGCTGTAGCCACT	CGGCTGATCATCATCTTG TG	877	3020	1821

**Table B4. List of primers used for gene expression analysis under abiotic stress conditions using RT-PCR.** Sequences of the primers presented as 5' to 3'.

<i>A. thaliana</i> gene	Abbreviation	Forward Primer	Reverse Primer	Product size (bp)
AT4G34890	<i>XDH1</i>	CGCGGATACACATGAAAATG	TTCCAGAGCTGATGCAATG	904
AT4G34900	<i>XDH2</i>	GTGGCTTTGGTGGTAAGGAA	CGTCTAAAACTGCAGCACCA	891
AT2G26230	<i>UO</i>	GAAAGCCAAGGAATGTGGAG	TGAAGGGTTTTCTTCGTTG	662
AT4G04955	<i>ALN</i>	GAACTAAGGCTGCTGCTGCT	TTCAGGTTCCACACAACAA	977
AT5G58220	<i>ALNS</i>	AATTCGCGAAGCAGATGTCTA	AGTGGGACATGGAAATGCTC	880
AT4G20070	<i>AAH</i>	AAGGGACGAAGCTGTAGCAA	TAGCACTTGGCCATGTTGAG	872
AT4G17050	<i>UGLYAH</i>	GCCCTTTACCTGACTGGACA	AGTCTTTCCGAGTGCAGCAT	611
AT5G43600	<i>UAH</i>	ATCTGCCTGCTGTAGCCACT	CGGCTGATCATCATCTTGTG	877
At5g09810	<i>ACTIN7</i>	GATATTCAGCCACTTGTCTGTGAC	CATGTTTCGATTGGATACTTCAGAG	187
At1g08830	<i>CSD1</i>	TGGCGAAAGGAGTTGCAGTT	TGGCAATCAGTGATTGTGAAG	304
AT4G35090	<i>CAT2</i>	TCAAGGAAGAAGGAGCTTTCA	CATCATGTGGATCTTCTGTGC	157
At1g07890	<i>APX1</i>	CCACTCGCATTTCTCCAGAT	TCGAAAGTTCCAGCAGAGTG	352
At4g27410	<i>RD26</i>	GATGTGAAGTTACTGATGGGTGAA	GCGAGCCAAGTCACAAGGAG	502
At5g52310	<i>RD29A</i>	ATGATGACGAGCTAGAACCTG	CCTTGTCGGATGTAAACG	228
At5g52310	<i>RD29B</i>	GAAGACAACGGCTACAAAGG	ACGGTGGTGCCAAGTGAT	219

**Table B5. List of primers used for qRT-PCR.** Sequences of the primers presented as 5' to 3'.

<i>A. thaliana</i> Gene	Name	Forward Primer	Reverse Primer	Product size (bp)
AT2G26230	<i>UO</i>	CACTGTTTATGTGAAAGCCAAGGAATG	CCCAAGCTTAAAACCATGTAAATGTGG	187
AT4G04955	<i>ALN</i>	CCTGGTCTCATTGATGTGCATGTC	TGTTTTTCGCAGCTTCAATCTTGAGT	187
AT3G18780	<i>ACTIN2</i>	TTGTGCTGGATTCTGGTGATGG	CCGCTCTGCTGTTGTGGTG	167
AT4G05320	<i>UBQ10</i>	TTTGTTAAGACTCTCACCGGAAAGACA	GAGGGTGGATTCCTTCTGGATATTGTA	192

**Table B6. Homozygous T-DNA insertion lines and the effect of insertion on the expression of the mutated gene detected by RT-PCR.** Genes are listed in order of appearance in the ureide metabolism pathway.

<b>T-DNA insertion line</b>	<b><i>A. thaliana</i> Gene</b>	<b>T-DNA insertion location</b>	<b>Transcript level of effected gene</b>
SALK_ 148366	<i>XDH1</i> (At4g34890)	Intron	No transcript
SALK_ 015081	<i>XDH2</i> (At4g34900)	Exon	No effect
SALK_ 096471	<i>UO</i> (At2g26230)	5' UTR	Reduced transcript
SALK_ 013986	<i>ALN</i> (At4g04955)	Exon	Reduced transcript
SAIL_ 810_E12	<i>ALN</i> (At4g04955)	Exon	No transcript
SALK_ 142607	<i>ALN</i> (At4g04955)	Intron	No effect
SALK_ 112631	<i>AAH</i> (At4g20070)	Intron	No transcript
SALK_ 071989	<i>UGLYAH</i> (At4g17050)	Exon	No effect
SALK_072119	<i>UGLYAH</i> (At4g17050)	Promoter	Increased transcript
SALK_ 108394	<i>UAH</i> (At5g43600)	Promoter	Increased transcript
SAIL_ 338_C08	<i>UAH</i> (At5g43600)	Exon	No transcript
SALK_ 024998	<i>UAH</i> (At5g43600)	Intron	No effect



**Table B7. Chlorophyll level (A),  $F_v/F_m$  ratio (B) and anthocyanin level (C) of Col-0 and *aln-3* leaves on day 0, 7, 14 and 21 after moderate light and increased light treatments.** Values represent the mean  $\pm$  SEM of three biological replicates. Significant difference between Col-0 and *aln-3* measurements for each light treatment and time point was determined by t-test (\*  $P < 0.05$ , \*\* $P < 0.01$ ).

A

Days	Moderate light intensity		Increased light intensity	
	<b>mg chlorophyll (g FW)<sup>-1</sup></b>		<b>mg chlorophyll (g FW)<sup>-1</sup></b>	
	Col-0	<i>aln-3</i>	Col-0	<i>aln-3</i>
0	0.70 $\pm$ 0.04	0.67 $\pm$ 0.04	-	-
7	0.73 $\pm$ 0.04	0.73 $\pm$ 0.05	0.54 $\pm$ 0.03	0.51 $\pm$ 0.03
14	0.85 $\pm$ 0.06	0.88 $\pm$ 0.07	0.33 $\pm$ 0.02	0.46 $\pm$ 0.02 **
21	0.64 $\pm$ 0.03	0.63 $\pm$ 0.03	0.06 $\pm$ 0.02	0.1 $\pm$ 0.01

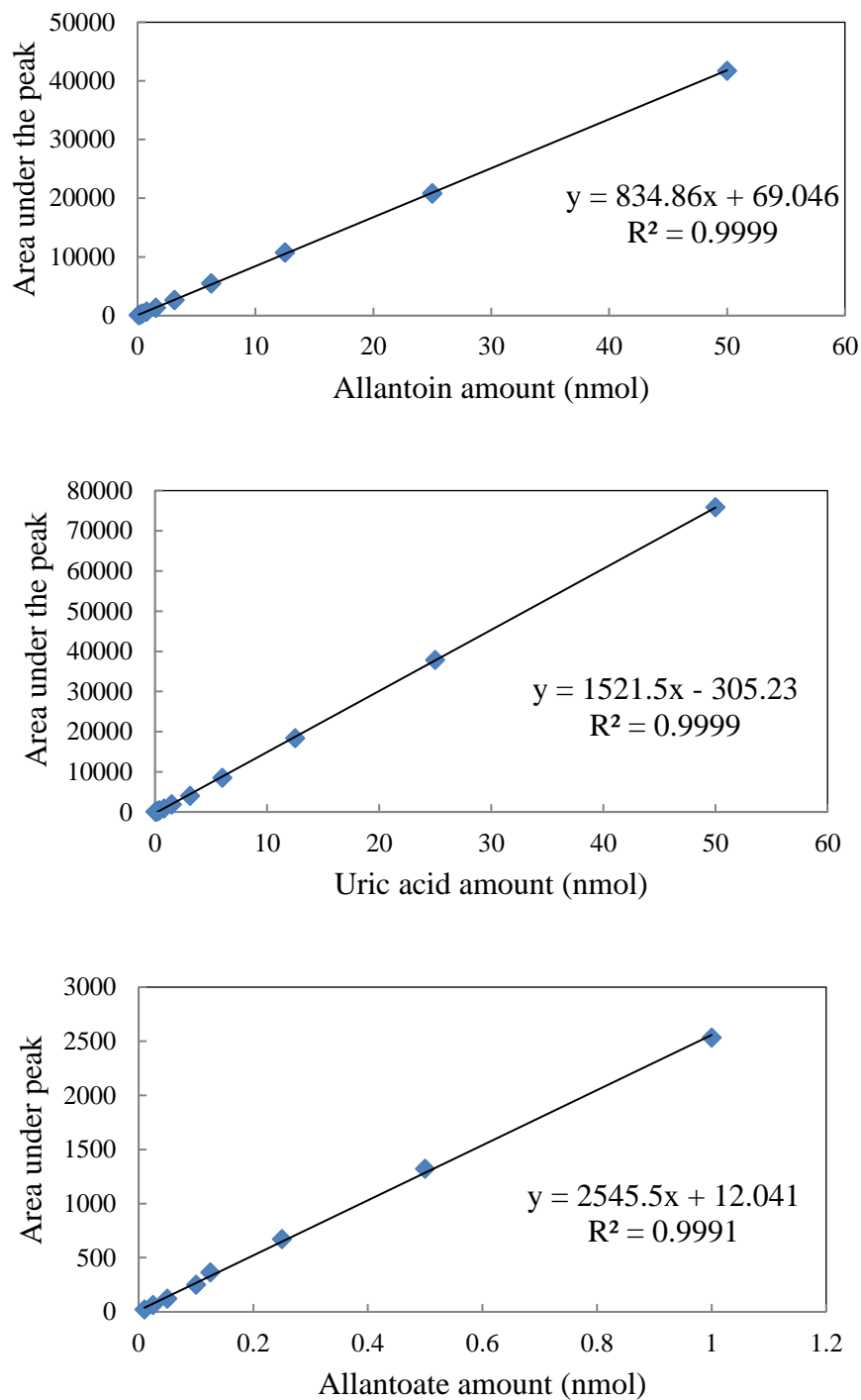
B

Days	Moderate light intensity		Increased light intensity	
	<b>(<math>F_v/F_m</math>)</b>		<b>(<math>F_v/F_m</math>)</b>	
	Col-0	<i>aln-3</i>	Col-0	<i>aln-3</i>
0	0.80 $\pm$ 0.00	0.80 $\pm$ 0.00	-	-
7	0.81 $\pm$ 0.01	0.81 $\pm$ 0.00	0.76 $\pm$ 0.01	0.76 $\pm$ 0.00
14	0.81 $\pm$ 0.00	0.81 $\pm$ 0.01	0.46 $\pm$ 0.01	0.57 $\pm$ 0.02 *
21	0.80 $\pm$ 0.01	0.80 $\pm$ 0.01	0	0

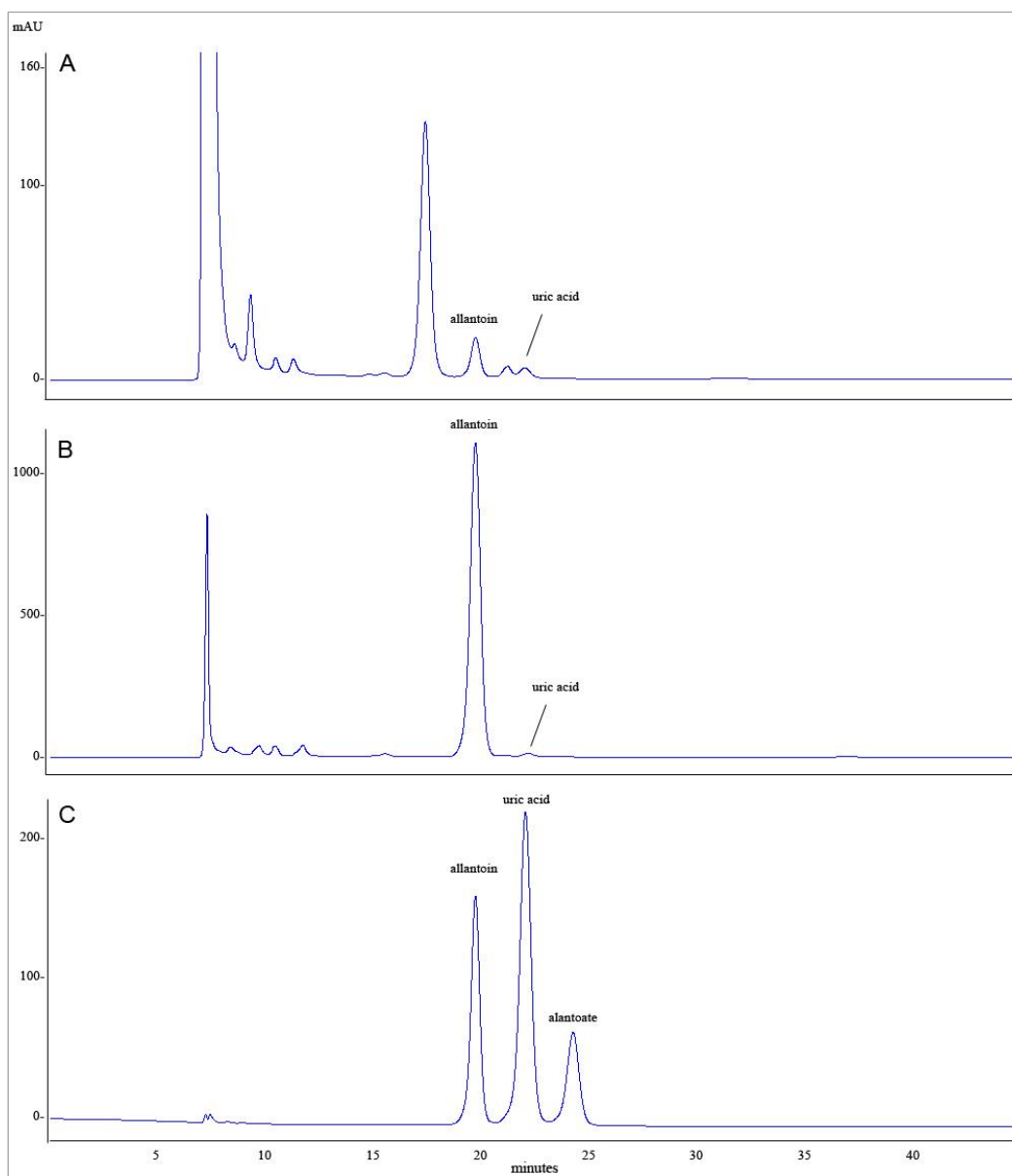
C

Days	Moderate light intensity		Increased light intensity	
	<b>Anthocyanin content (abs. /g FW)<sup>-1</sup></b>		<b>Anthocyanin content (abs. /g FW)<sup>-1</sup></b>	
	Col-0	<i>aln-3</i>	Col-0	<i>aln-3</i>
0	0	0	-	-
7	0.31 $\pm$ 0.02	0.28 $\pm$ 0.02	2.5 $\pm$ 0.3	2.3 $\pm$ 0.3
14	0.53 $\pm$ 0.03	0.48 $\pm$ 0.05	35 $\pm$ 1.1	28 $\pm$ 0.1 **
21	0.78 $\pm$ 0.02	0.80 $\pm$ 0.03	65 $\pm$ 0.6	61 $\pm$ 0.7 *

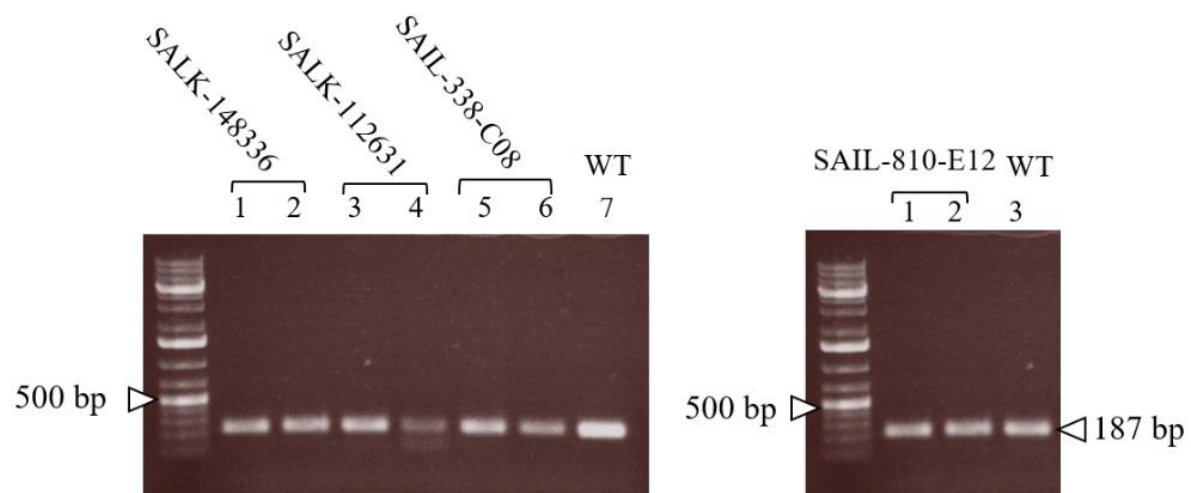
## 7. APPENDIX C. Supplementary figures



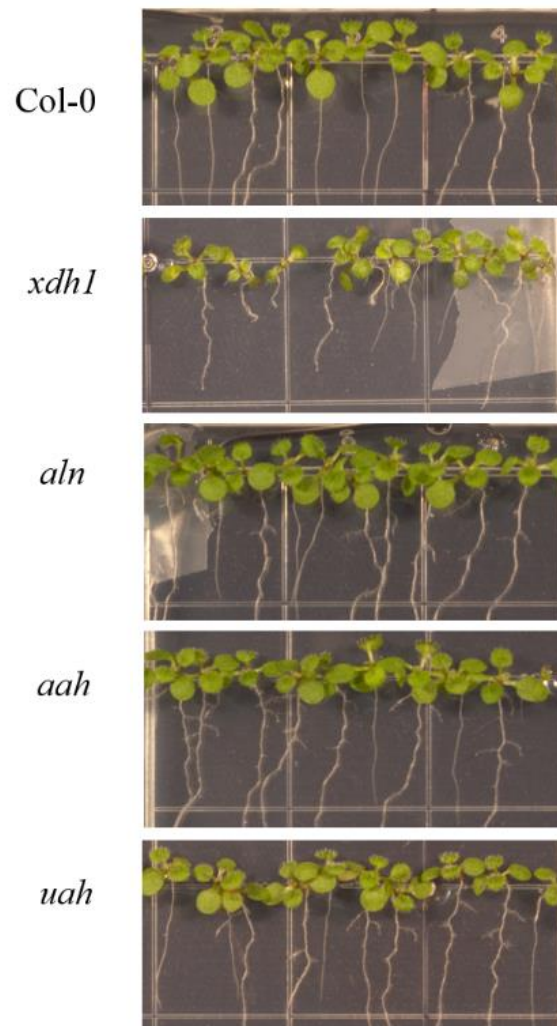
**Figure C1. HPLC Standard calibration curves for allantoin, uric acid and allantoate.**



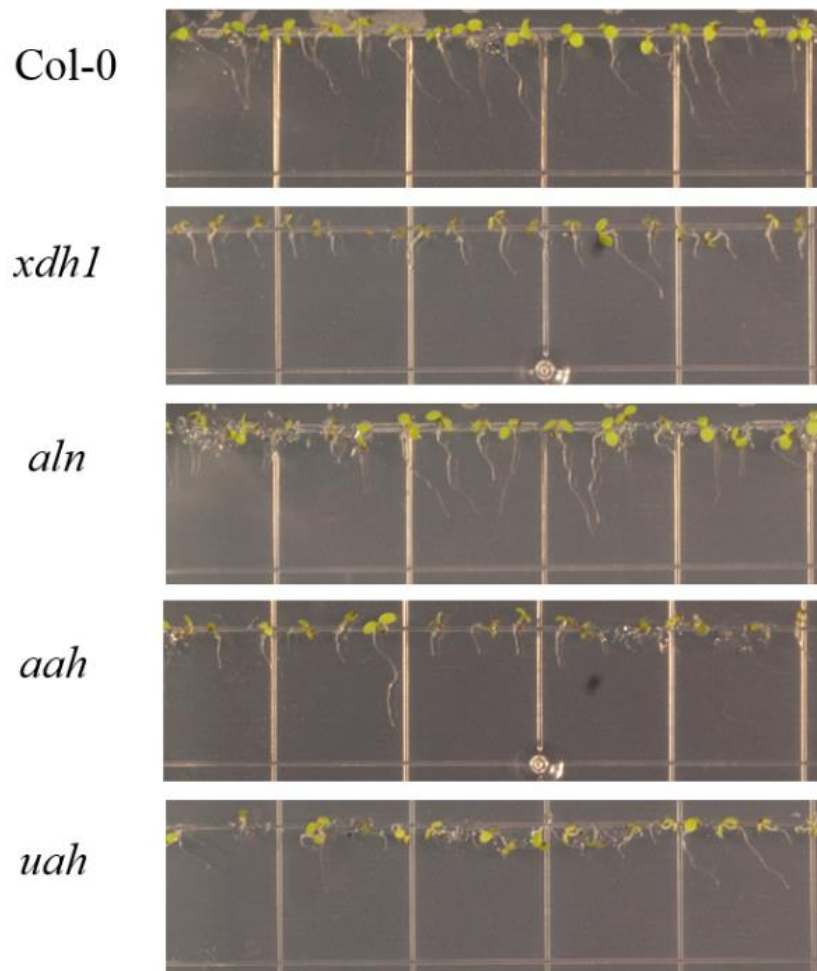
**Figure C2. HPLC separation of ureides.** Typical chromatograms from Col-0 (A) and *aln-3* (B) leaf tissue samples, as well as a mixed standard containing pure allantoin, allantoate, and uric acid (C).



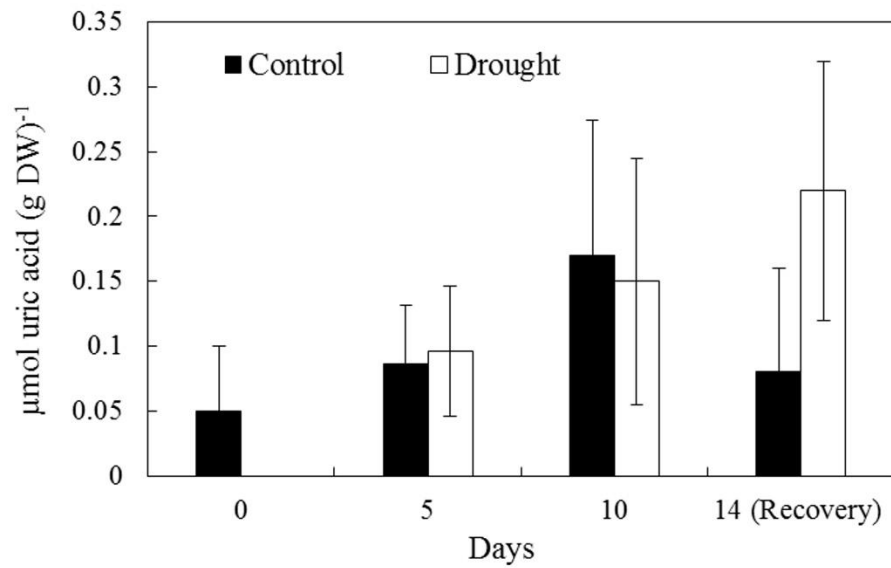
**Figure C3. Control RT-PCR analysis for four homozygous mutants without expression of the mutant genes. *ACTIN7* (187 bp) was used as a control.**



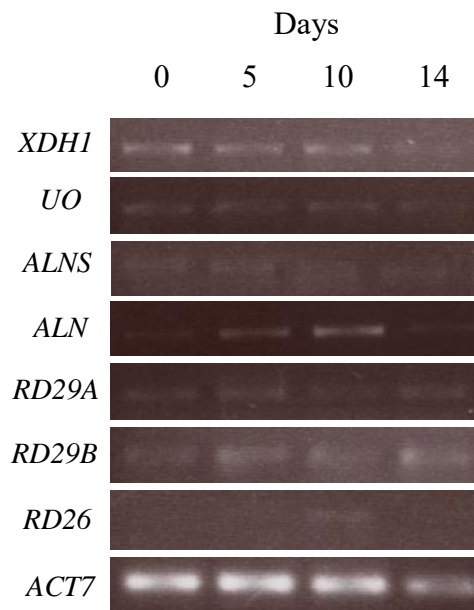
**Figure C4. Phenotype of Col-0 and mutant seedlings when seeds were germinated on MS control.** The picture was taken 14 days after transfer to germination-permissive conditions.



**Figure C5. Phenotype of Col-0 and mutant seedlings when the seeds were germinated on 150 mM NaCl.** The picture was taken after 10 days under germination-permissive conditions.



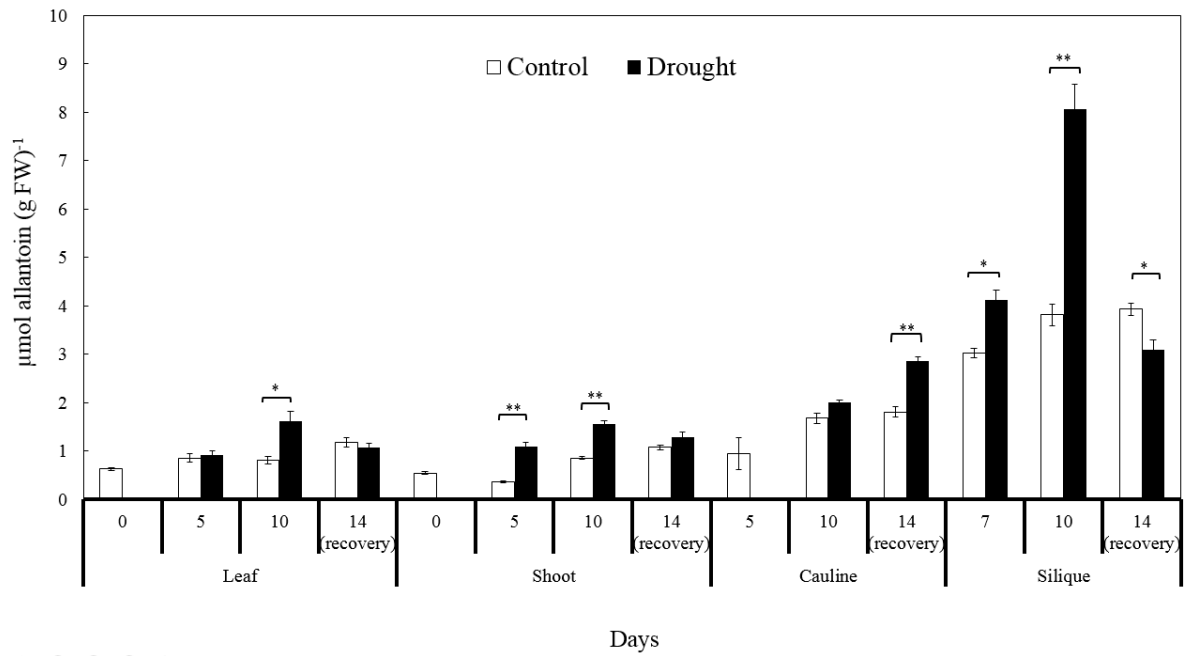
**Figure C6. Uric acid level in Col-0 leaves in response to drought and well-watered conditions.** Uric acid content in Col-0 leaves after 5 and 10 days of withholding water and on day 14 which represents two days after re-watering drought-stressed plants (white bars) and well-watered control condition (black bars). Day 0 indicates the day before withholding water. DW, dry weight. Error bars are the standard error of the mean (SEM). (n= 3).



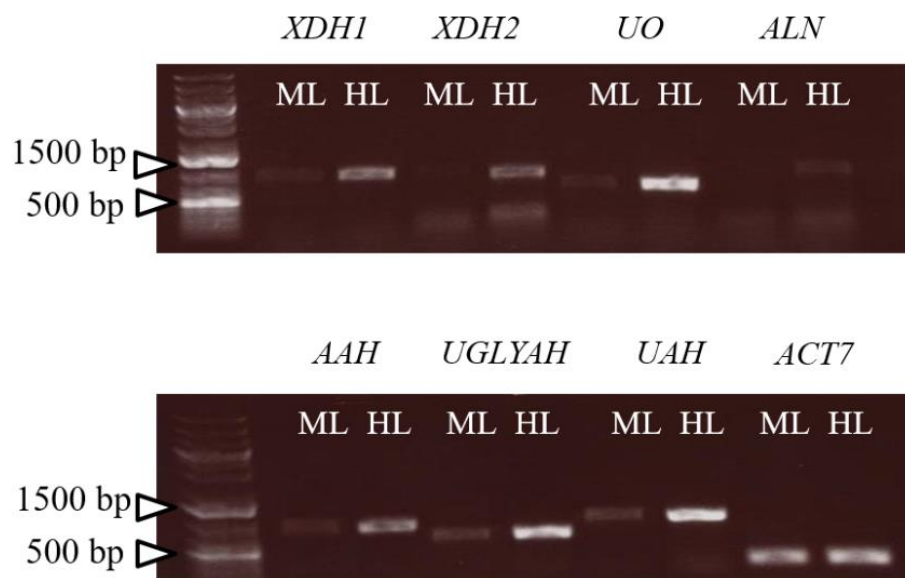
**Figure C7. RT-PCR analysis of a number of ureide metabolic genes under well-watered conditions.**

Expression analysis of a number of ureide metabolism genes in leaf tissue of Col-0 plants on day 0, 5, 10 and 14 days well-watered (control for drought stress) plants. *RD29A*, *RD29B* and *RD26* represent stress-responsive genes and *ACT7* served as reference gene. *XDHI*, xanthine dehydrogenase1; *UO*, uricase; *ALNS*, allantoin synthase; *ALN*, allantoinase; *RD*, dehydration-responsive genes; *ACT7*, Actin7. Image is representative of three independent replicates.

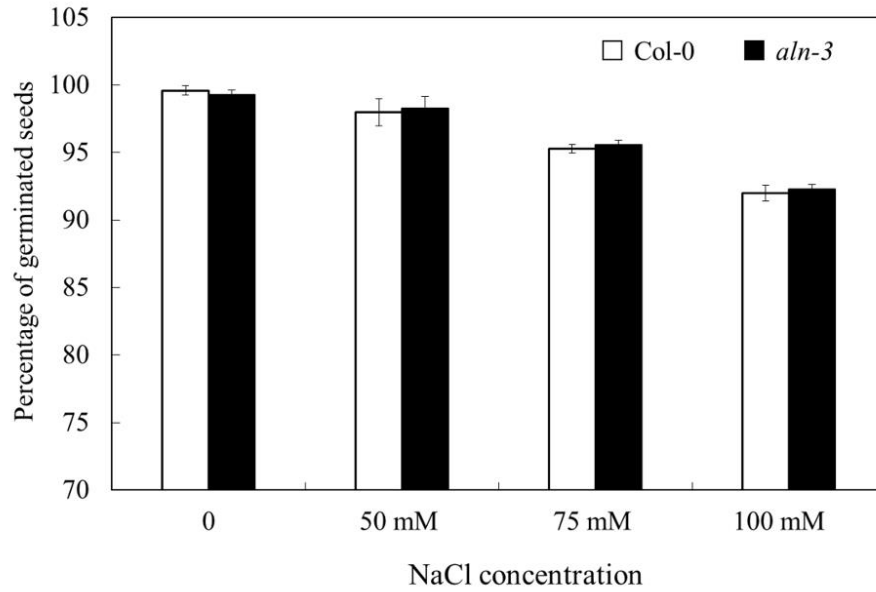




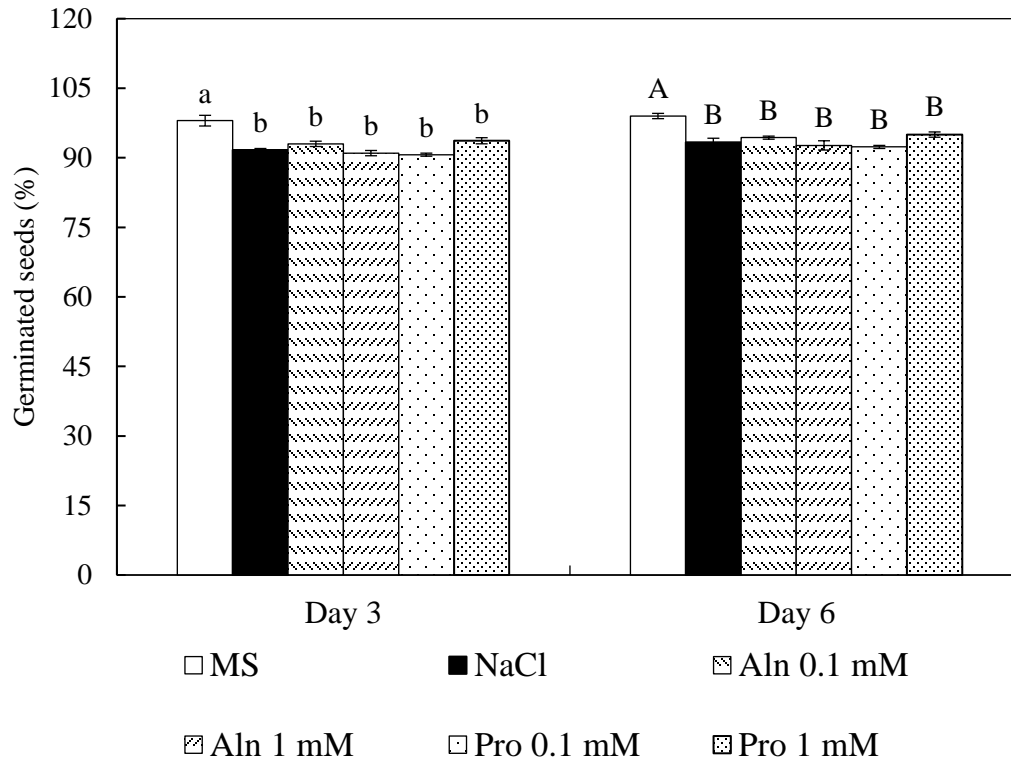
**Figure C8. Allantoin content in *aln-3* in response to drought and control conditions.** Accumulation of allantoin in various tissues of *aln-3* after 0, 5 and 10 days of non-stress condition (white bars) and drought stress (black bars). Day 14 represents 2 days after re-watering. Cauline leaves and siliques started to form on day 5 and 7, respectively. Data presented are the mean of three independent replicates  $\pm$  SEM. Asterisks indicate significant differences determined by a t-test between drought and control data on the same time points (\*  $P < 0.05$ , \*\* $P < 0.01$ ). (n= 3)



**Figure C9. RT-PCR analysis of ureide metabolism genes in Col-0 leaves, 10 days after moderate or increased light treatments.** 17-day-old plants were shifted to chambers with moderate or increased light conditions. In order to analyze the expression of some ureide metabolism genes, RNA was extracted from leaf tissue of Col-0 plants 10 days after moderate or increased light treatments. *XDH*, xanthine dehydrogenase; *UO*, uricase; *ALN*, allantoinase; *AAH*, allantoate amidohydrolase; *UGLYAH*, ureidoglycine aminohydrolase; *UAH*, ureidoglycolate amidohydrolase; *ACT7*, Actin7. *ACT7* served as reference gene. (n= 2)



**Figure C10. Percentage of germinated Col-0 and *aln-3* seeds, three days after transfer to germination-permissive conditions.** No significant difference was found between the germination percentage of Col-0 and *aln-3* seeds on each media when tested by t-test at  $p < 0.05$ . Two-way-ANOVA analysis of data confirmed that there is no significant impact of genotypes but the effect of treatments (NaCl concentrations) is significant at  $p < 0.05$ .



**Figure C11. Percentage of germinated Col-0 seeds, three and six days after transfer to germination-permissive conditions.** ALN: allantoin; Pro: proline; MS represents the media without NaCl as control. NaCl media contained 100 mM NaCl. All four media of ALN 0.1 mM, ALN 1 mM, Pro 0.1 mM and Pro 1 mM contained 100 mM NaCl. Measurements were made of at least 30 seedlings per experiment. Data presented are the mean of three independent replicates  $\pm$  SEM. Tukey's test was used to compare the means of each treatment to the means of other treatments. Different letters above the bars indicate significant differences detected by Tukey HSD test at  $p < 0.05$ . (n= 3).

## 8. REFERENCES

- Abeles FB, Morgan PW, Saltveit ME. (1992) Ethylene in plant biology. San Diego, CA, USA: Academic Press.
- Agati G, Azzarello E, Pollastri S, Tattini M. (2012) Flavonoids as antioxidants in plants: location and functional significance. *Plant Sci.* 196: 67-76.
- Agrawal GK, Bourguignon J, Rolland N, Ephritikhine G, Ferro M, Jaquinod M, Alexiou KG, Chardot T, Chakraborty N, Jolivet P, Doonan JH, Rakwal R. (2011) Plant organelle proteomics: Collaborating for optimal cell function. *Mass Spectrom Rev.* 30: 772-853.
- Ahmad P, Prasad MNV. (2012) Abiotic stress responses in plants: Metabolism, productivity and sustainability. Springer, New York. pp 1-19.
- Ahmad P, Rasool S. (2014) Emerging technologies and management of crop stress tolerance. Volume 1. Biological techniques. London: Elsevier, Academic press. pp 99-123.
- Akinci S. (1997). Physiological responses to water stress by *Cucumis sativus* L. and related species. PhD thesis. University of Sheffield. Department of Animal and Plant Sciences. England.
- Alamillo JM, Díaz-Leal JL, Sánchez-Moran MV, Pineda M. (2010) Molecular analysis of ureide accumulation under drought stress in *Phaseolus vulgaris* L. *Plant Cell Environ.* 33: 1828-1837.
- Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P, Tiburcio AF. (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta.* 231: 1237-1249.
- Alexandersson E, Saalbach G, Larsson C, Kjellbom P. (2004) Arabidopsis plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. *Plant Cell Physiol.* 45: 1543-1556.
- Alvarez S, Marsh EL, Schroeder SG, Schachtman DP. (2008) Metabolomic and proteomic changes in the xylem sap of maize under drought. *Plant Cell Environ.* 31: 325–340.
- Ames BN, Cathcart R, Schwiers E, Hochstein P. (1981) Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: A hypothesis. *Proc Natl Acad Sci U S A.* 78: 6858-6862.

- Amudha J, Balasubramani G. (2011) Recent molecular advances to combat abiotic stress tolerance in crop plants. *Biotechnol Mol Biol Rev.* 6: 31-58.
- Argyrou E, Sophianopoulou V, Schultes N, Diallynas G. (2001). Functional characterization of a maize purine transporter by expression in *Aspergillus nidulans*. *Plant Cell.* 13: 953-964.
- Armbruster U, Hertle A, Makarenko E, Zühlke J, Pribil M, Dietzmann A, Schliebner I, Aseeva E, Fenino E, Scharfenberg M, Voigt C, Leister D. (2009) Chloroplast proteins without cleavable transit peptides: rare exceptions or a major constituent of the chloroplast proteome? *Mol Plant.* 2: 1325-1335.
- Aroca R, Porcel R, Ruiz-Lozano JM. (2012) Regulation of root water uptake under abiotic stress conditions. *J Exp Bot.* 63: 43-57.
- Asada K. (1999) The water-water cycle in chloroplast: scavenging of active oxygens and dissipation of Excess Photons. *Annu Rev Plant Physiol.* 50: 601-639.
- Ashraf M, Foolad MR. (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ Exp Bot.* 59: 206-216.
- Ashraf M, Harris PJC. (2013) Photosynthesis under stressful environments: An overview. *Photosynthetica.* 51: 163-190.
- Atkins CA, Pate JS, Ritchie A, Peoples MB. (1982) Metabolism and translocation of allantoin in ureide-producing grain legumes. *Plant Physiol.* 70:476-482.
- Barber J. (1995) Molecular basis of the vulnerability of photosystem II to damage by light. *Aust J Plant Physiol.* 22: 201-208.
- Bachrach U. (2010) The early history of polyamine research. *Plant Physiol Bioch.* 48: 490-495.
- Bailey S, Walters RG, Jansson S, Horton P. (2001) Acclimation of *Arabidopsis thaliana* to the light environment: The existence of separate low light and high light responses. *Planta.* 213: 794-801.
- Beggs JD. (1978) Transformation of yeast by a replicating hybrid plasmid. *Nature.* 275: 104-109.

Ben Ahmed C, Ben Rouina B, Sensoy S, Boukhriss M, Ben Abdullah F. (2010) Exogenous proline effects on photosynthetic performance and antioxidant defense system of young olive tree. *J Agric Food Chem.* 58: 4216-4222.

Bhattacharjee S. (2005) Reactive oxygen species and oxidative burst: Roles in stress, senescence and signal transduction in plants. *Curr Sci.* 89: 1113-1121.

Blelski BHJ, Shiue GG, Bajuk S. (1980) Reduction of nitro blue tetrazolium by  $\text{CO}_2^-$  and  $\text{O}_2^-$  radicals. *J Phys Chem.* 84: 830-833.

Bohnert HJ, Nelson DE, Jensen RG. (1995) Adaptations to environmental stresses. *Plant Cell.* 7: 1099-1111.

Bollard EG. (1956) Nitrogenous compounds in plant xylem sap. *Nature.* 178: 1189-1190.

Borrell AK, Hammer GL, Henzell RG. (2000) Does maintaining green leaf area in sorghum improve yield under drought? II. Dry matter production and yield. *Crop Sci.* 40: 1037-1048.

Boyer JS. (1982). Plant productivity and environment. *Science.* 218: 443-448.

Bowler C, Van Camp W, Van Montagu M, Inze D, Asada k. (1994) Superoxide dismutase in plants. *Crit Rev Plant Sci U S A.* 13: 199-218.

Brychkova G, Xia Z, Yang G, Yesbergenova Z, Zhang Z, Davydov O, Fluhr R, Sagi M. (2007) Sulfite oxidase protects plants against sulfur dioxide toxicity. *Plant J.* 50: 696-709.

Brychkova G, Alikulov Z, Fluhr R, Sagi M. (2008) A critical role for ureides in dark and senescence-induced purine remobilization is unmasked in the *Atxdh1* Arabidopsis mutant. *Plant J.* 54: 496-509.

Caldwell CR, Whitman CE. (1987). Temperature-induced protein conformational changes in barley root plasma membrane-enriched microsomes. I. Effect of temperature on membrane protein and lipid mobility. *Plant Physiol.* 84: 918-923.

Carlson M, Botstein D. (1982) Two differentially regulated mRNAs with different 5' ends encode secreted with intracellular forms of yeast invertase. *Cell.* 28: 145-154.

Castro AHF, Young MCM, De Alvarenga AA, Alves JD. (2001) Influence of photoperiod on the accumulation of allantoin in comfrey plants. *Rev Bras Fisiol Veg.* 13: 49-54.

Chalker-Scott L. (1999) Environmental significance of anthocyanins in plant stress responses. *Photochem Photobiol.* 70: 1-9.

Chalker-Scott L, Fenchigami LH. (1989) The role of phenolic compounds in plant stress responses. In: Paul HL, Ed. Low temperature stress physiology in crops. Boca Raton, Florida: CRC Press Inc. pp 40.

Chang YC, Lee FW, Chen CS, Huang ST, Tsai SH, Huang SH, Lin CM. (2007) Structure–activity relationship of C6–C3 phenylpropanoids on xanthine oxidase-inhibiting and free radical-scavenging activities. *Free Radical Biol Med.* 43: 1541-1551.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. (1994) Green fluorescent protein as a marker for gene expression. *Science.* 263: 802-805.

Chaves MM, Flexas J, Pinheiro C. (2009) Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. *Ann Bot.* 103: 551-560.

Chen TH, Murata N. (2008) Glycinebetaine: An effective protectant against abiotic stress in plants. *Trends Plant Sci.* 13: 499-505.

Chen TH, Murata N. (2011) Glycinebetaine protects plants against abiotic stress: Mechanisms and biotechnological applications. *Plant Cell Environ.* 34: 1-20.

Christensen T, Jochimsen BU. (1983) Enzymes of ureide synthesis in pea and soybean. *Plant Physiol.* 72: 56-59.

Coleto I, Pineda M, Rodiño AP, De Ron AM, Alamillo JM. (2014) Comparison of inhibition of N<sub>2</sub> fixation and ureide accumulation under water deficit in four common bean genotypes of contrasting drought tolerance. *Ann Bot.* 113: 1071-1082.

Collier R, Tegeder M. (2012) Soybean ureide transporters play a critical role in nodule development, function and nitrogen export. *Plant J.* 72: 355-367.

Corby HDL. (1988) Types of rhizobial nodule and their distribution among the Leguminosae. *Kirkia.* 13: 53-123.



- Corpas FJ, De la Colina C, S  nchez-Rasero F, Del Rio LA. (1997) A role for leaf peroxisomes in the catabolism of purines. *J Plant Physiol.* 151: 246-250.
- Dabrowska G, Kata A, Goc A, Szechynska-Hebda M, Skrzyek E. (2007) Characteristics of the ant ascorbate peroxidase family. *Acta Biol Cracoviensia Ser Bot.* 49: 7-17.
- Dahncke K, Witte CP. (2013) Plant purine nucleoside catabolism employs a guanosine deaminase required for generation of xanthosine in Arabidopsis. *Plant Cell.* 25: 4101-4109.
- Daneshmand F, Arvin MJ, Kalantari KM. (2010) Physiological responses to NaCl stress in three wild species of potato *in vitro*. *Acta Physiol Plant.* 32: 91-101.
- Dantas BF, de S   Ribeiro L, Arag  o CA. (2007) Germination, initial growth and cotyledon protein content of bean cultivars under salinity stress. *Rev Bras Sementes.* 29: 106-110.
- Davies WJ, Wilkinson S, Loveys B. (2002) Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture. *New Phytol.* 153: 449-460.
- Day DA, Copeland L. (1991) Carbon metabolism and compartmentation in nitrogen-fixing legume nodules. *Plant Physiol Biochem.* 29: 185-201.
- Debnath M, Pandey M, Bisen PS. (2011) An omics approach to understand the plant abiotic stress. *OMICS.* 15: 739-762.
- Degenkolbe T, Do PT, Kopka J, Zuther E, Hinch DK, K  hl KI. (2013) Identification of drought tolerance markers in a diverse population of rice cultivars by expression and metabolite profiling. *PLoS One.* 8: e63637.
- Deinlein U, Stephan AB, Horie T, Luo W, Xu G, Schroeder JI. (2014). Plant salt-tolerance mechanisms. *Trends Plant Sci.* 19: 371-379.
- Deng Y, Srivastava R, Howell SH. (2013) Endoplasmic reticulum (ER) stress response and its physiological roles in plants. *Int J Mol Sci.* 14: 8188-8212.
- Del R  o LA, Corpas FJ, Sandalio LM, Palma JM, Barroso JB. (2003) Plant peroxisomes, reactive oxygen metabolism and nitric oxide. *IUBMB Life.* 55: 71-81.

- Demmig-Adams B, Adams WW 3<sup>rd</sup>. (2006) Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. *New Phytol.* 172: 11-21.
- Desimone M, Catoni E, Ludewig U, Hilpert M, Schneider A, Kunze R, Tegeder M, Frommer WB, Schumacher K. (2002) A novel superfamily of transporters for allantoin and other oxo derivatives of nitrogen heterocyclic compounds in Arabidopsis. *Plant Cell.* 14: 847-856.
- De Silva M, Purcell L, King CA. (1996) Soybean petiole ureide response to water deficits and decreased transpiration. *Crop Sci.* 36: 611-616.
- Divi UK, Krishna P. (2009) Brassinosteroid: a biotechnological target for enhancing crop yield and stress tolerance. *N Biotechnol.* 26: 131-136.
- Dixon RA, Paiva N. (1995) Stressed induced phenyl propanoid metabolism. *Plant Cell.* 7: 1085-1097.
- Doyle J, Doyle J, Ballenger J, Dickson E, Kajita T, Ohashi H. (1997) Phylogeny of the chloroplast gene RBCL in the Leguminosae: taxonomic correlations and insights into the evolution of nodulation. *Am J Bot.* 84: 541-554.
- Du H, Liu H, Xiong L. (2013) Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. *Front Plant Sci.* 4: 397-405.
- Edwards K, Johnstone C, Thompson C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* 19: 1349-1356.
- Einset J, Nielsen E, Connolly EL, Bones A, Sparstad T, Winge P, Zhu JK. (2007) Membrane-trafficking RabA4c involved in the effect of glycine betaine on recovery from chilling stress in Arabidopsis. *Physiol Plant.* 130: 511-518.
- Engelbrecht L. (1955) Über allantoinsäure und allantoin. IV. Ihre beziehungen zu den säureamiden bei der keimung von *Phaseolus vulgaris* L. *Flora Oder Allgemeine Botanische Zeitung.* 142: 25-44.
- Fang Y, Xiong L. (2015) General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell Mol Life Sci.* 72: 673-689.
- Fernández AP, Strand A. (2008) Retrograde signaling and plant stress: Plastid signals initiate cellular stress responses. *Curr Opin Plant Biol.* 11: 509-513.

- Fett-Neto AG, Pennington JJ, Di Cosmo F. (1995) Effect of white light on taxol and baccatin III accumulation in cell cultures of *Taxus cuspidata* sieb and zucc. *J Plant Physiol.* 146: 584-590.
- Feussner I, Wasternack C. (2002). The lipoxygenase pathway. *Annu Rev Plant Biol.* 53: 275-297.
- Finkelstein RR, Gampala SSL, Rock CD. (2002) Absciscic acid signaling in seeds and seedlings. *Plant Cell.* 14: S15-S45.
- Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L. (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature.* 422: 442-446.
- Foyer CH, Lelandais M, Kunert KJ. (1994) Photooxidative stress in plants. *Physiol Plant.* 92: 696-717.
- Foyer CH, Noctor G. (2009) Redox regulation in photosynthetic organisms: Signaling, acclimation, and practical implications. *Antioxid Redox Signal.* 11: 861-905.
- Fromme P, Jordan P, Krauss N. (2001) Structure of photosystem I. *Biochim Biophys Acta.* 1507: 5-31.
- Frugoli JA, Zhong HH, Nuccio ML, McCourt P, McPeck MA, Thomas TL, McClung CR. (1996) Catalase is encoded by a multigene family in *Arabidopsis thaliana* (L.) Heynh. *Plant Physiol.* 112: 327-336.
- Fujihara S, Yamaguchi M. (1978) Effects of allopurinol [4-hydroxyprazolo(3, 4-d)pyrimidine] on the metabolism of allantoin in soybean plants. *Plant Physiol.* 62: 134-138.
- Fujii H, Verslues PE, Zhu JK. (2011) Arabidopsis decuple mutant reveals the importance of SnRK2 kinases in osmotic stress responses *in vivo*. *Proc Natl Acad Sci U S A.* 108: 1717-1722.
- Fujita M, Mizukado S, Fujita Y, Ichikawa T, Nakazawa M, Seki M, Matsui M, Yamaguchi-Shinozaki K, Shinozaki K. (2007) Identification of stress-tolerance-related transcription-factor genes via mini-scale Full-length cDNA Over-eXpressor (FOX) gene hunting system. *Biochem Biophys Res Commun.* 364: 250-257.
- Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LS, Yamaguchi-Shinozaki K, Shinozaki K. (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. *Plant J.* 39: 863-876.

- Gapper C, Dolan L. (2006) Control of plant development by reactive oxygen species. *Plant Physiol.* 141: 341-345.
- Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR. (2001) Drought- and salt- tolerant plants result from overexpression of the AVP1 H<sup>+</sup>-pump. *Proc Natl Acad Sci U S A.* 98:11444-11449.
- Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C. (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bioessays.* 28: 1091-1101.
- Gest N, Gautier H, Stevens R. (2013) Ascorbate as seen through plant evolution: the rise of a successful molecule? *J Exp Bot.* 64: 33-53.
- Gill SS, Tuteja N. (2010a) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem.* 48: 909-930.
- Gill SS, Tuteja N. (2010 b) Polyamines and abiotic stress tolerance in plants. *Plant Signal Behav.* 5: 26-33.
- Gillissen B, Bürkle L, André B, Kühn C, Rentsch D, Brandl B, Frommer WB. (2000). A new family of high-affinity transporters for adenine, cytosine, and purine derivatives in Arabidopsis. *Plant Cell.* 12: 291-300.
- Golldack D, Li C, Mohan H, Probst N. (2014) Tolerance to drought and salt stress in plants: Unraveling the signaling networks. *Front Plant Sci.* 5: 151-157.
- Gould KS. (2004) Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. *J Biomed Biotechnol.* 2004: 314-320.
- Gray GR, Chauvin LP, Sarhan F, Huner N. (1997) Cold Acclimation and Freezing Tolerance (A Complex Interaction of Light and Temperature). *Plant Physiol.* 114: 467-474.
- Gray GR, Savitch LV, Ivanov AG, Huner N. (1996) Photosystem II Excitation Pressure and Development of Resistance to Photoinhibition (II. Adjustment of Photosynthetic Capacity in Winter Wheat and Winter Rye). *Plant Physiol.* 110: 61-71.

Grune T, Schröder P, Biesalski HK. (2005) Low molecular weight antioxidants. The Handbook of Environmental chemistry. Vol.2. Berlin Heidelberg: Springer-Verlag. pp 77–90.

Gururani MA, Venkatesh J, Tran LS. (2015) Regulation of photosynthesis during abiotic stress-induced photoinhibition. *Mol Plant*. 8: 1304-1320.

Gus'kov EP, Shkurat TP, Milyutina NP, Prokof'ev VN, Pokudina IO, Mashkina EV, Timofeeva IV. (2001) Effect of allantoin on the activity of enzymes providing regulation of the ROS-dependent status of an organism. *Dokl Biochem Biophys*. 379: 239-242.

Gus'kov EP, Prokof'ev VN, Kletskii ME, Kornienko IV, Gapurenko OA, Olekhovich LP, Chistyakov VA, Shestopalov AV, Sazykina MA, Markeev AV, Shkurat TP, Malkhos'yan SR, Zhdanov YA. (2004) Allantoin as a vitamin. *Dokl Biochem Biophys*. 398: 320-324.

Hanks JF, Tolbert NE, Schubert KR. (1981) Localization of enzymes of ureide biosynthesis in peroxisomes and microsomes of nodules. *Plant Physiol*. 68: 65-69.

Hartmann VE, Arnold G. (1974) Über den ureidstoffwechsel beim laubmoosprotonema von *Funaria hygrometrica* L. (SIBTH.). III. Einfluss von licht und dunkelheit auf die aufnahme und den umsatz von allantoin-7-14C. *Biochemie und Physiologie der Pflanzen*. 166: 57-72.

Hartung W, Sauter A, Hose E. (2002) Absciscic acid in the xylem: Where does it come from, where does it go to? *J Exp Bot*. 53: 27-37.

Hauck OK, Scharnberg J, Escobar NM, Wanner G, Giavalisco P, Witte CP. (2014) Uric acid accumulation in an *Arabidopsis* urate oxidase mutant impairs seedling establishment by blocking peroxisome maintenance. *Plant Cell*. 26: 3090-3100.

Havaux M. (2014) Carotenoid oxidation products as stress signals in plants. *Plant J*. 79: 597-606.

Hesberg C, Hänsch R, Mendel RR, Bittner F. (2004) Tandem orientation of duplicated xanthine dehydrogenase genes from *Arabidopsis thaliana*: differential gene expression and enzyme activities. *J Biol Chem*. 279: 13547-13554.

Hajdukiewicz P, Svab Z, Maliga P. (1994) The small versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol Biol*. 25: 989-994.

- Halliwell B. (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol.* 141: 312-322.
- Hanson MR, Kohler RH. (2001) GFP imaging: Methodology and application to investigate cellular compartmentation in plants. *J Exp Bot.* 52: 529-539.
- Hays DB, Do JH, Mason RE, Morgan G, Finlayson SA. (2007) Heat stress induced ethylene production in developing wheat grains induces kernel abortion and increased maturation in a susceptible cultivar. *Plant Sci.* 172: 1113-1123.
- Hediger MA, Johnson RJ, Miyazaki H, Endou H. (2005) Molecular physiology of urate transport. *Physiology.* 20: 125-133.
- Herczenik E, Gebbink MF. (2008). Molecular and cellular aspects of protein misfolding and disease. *FASEB J.* 22: 2115-2133.
- Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant CIS-acting regulatory DNA elements (PLACE) database. *Nucleic Acids Res.* 27:297-300.
- Hinnen A, Hicks JB, Fink GR. (1978) Transformation of yeast. *Proc Natl Acad Sci U S A.* 75: 1929-1933.
- Hirayama T, Shinozaki K. (2010) Research on plant abiotic stress responses in the post-genome era: past, present and future. *Plant J.* 61: 1041-1052
- Ho MW, Ryan A, Cummins J. (1999) Cauliflower mosaic virus promoter – a recipe for disaster. *Microb Ecol Health Dis.* 10: 33-59.
- Hoque MA, Okuma E, Banu MN, Nakamura Y, Shimoishi Y, Murata Y. (2007) Exogenous proline mitigates the detrimental effects of salt stress more than exogenous betaine by increasing antioxidant enzyme activities. *J Plant Physiol.* 164: 553-561.
- Hoque MA, Banu MN, Nakamura Y, Shimoishi Y, Murata Y. (2008) Proline and glycinebetaine enhance antioxidant defense and methylglyoxal detoxification systems and reduce NaCl-induced damage in cultured tobacco cells. *J Plant Physiol.* 165: 813-284.
- Hong JH, Seah SW, Xu J. (2013) The root of ABA action in environmental stress response. *Plant Cell Rep.* 32: 971-983.

- Hossain MA, Bhattacharjee S, Armin SM, Qian P, Xin W, Li HY, Burritt DJ, Fujita M, Tran LS. (2015) Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. *Front Plant Sci.* 6:420-427.
- Huang B (2006) Plant-environment interactions. Third Edition. Boca Raton, Florida: CRC Press. pp 5-27
- Hull R, Covey SN, Dale P. (2000) Genetically modified plants and the 35S promoter: Assessing the risks and enhancing the debate. *Microb Ecol Health Dis.* 12: 1-5.
- Huner NPA, Öquist G, Sarhan F. (1998) Energy balance and acclimation to light and cold. *Trends Plant Sci.* 3: 224-230.
- Igarashi K, Kashiwagi K. (2010) Modulation of cellular function by polyamines. *Intl J Biochem Cell Biol.* 42: 39-51.
- Il'yasova D, Spasojevic I, Wang F, Tolun AA, Base K, Young SP, Marcom PK, Marks J, Mixon G, DiGiulio R, Millington DS. (2010) Urinary biomarkers of oxidative status in a clinical model of oxidative assault. *Cancer Epidemiol Biomarkers Prev.* 19: 1506-1510.
- Ishizuka JF, Okino, Hoshi S. (1970) Physiological studies on the nutrition of soybean plants .III. The relation between contents of nitrogenous components in stems and vegetative growth. *Soil Sci Plant Nutr.* 41: 78-82.
- Itai C, Benzioni A, Ordin L. (1973) Correlative changes in endogenous hormone levels and shoot growth induced by short heat-treatments to root. *Physiol Plant.* 29: 355-360.
- James RA, Caemmerer S, Condon AG, Zwart AB, Munns R. (2008) Genetic variation in tolerance to the osmotic stress component of salinity stress in durum wheat. *Funct Plant Biol.* 35: 111-123.
- Janiak A, Kwaśniewski M, Szarejko I. (2016) Gene expression regulation in roots under drought. *J Exp Bot.* 67: 1003-1014.
- Jaspers P, Kangasjarvi J. (2010) Reactive oxygen species in abiotic stress signaling. *Physiol Plant.* 138: 405-413.
- Jefferson RA, Burgess SM, Hirsht D. (1986) beta-Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc Natl Acad Sci. U S A.* 83: 8447-8451.

Jefferson RA, Kavanagh TA, Bevan MW. (1987) GUS fusions: B-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901-3907.

Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kim C, Jang S, Yang K, Nam J, An K, Han MJ, Sung RJ, Choi HS, Yu JH, Choi JH, Cho SY, Cha SS, Kim SI, An G. (2000) T-DNA insertional mutagenesis for functional genomics in rice. *Plant J.* 22: 561-570.

Ji H, Pardo JM, Batelli G, Van Oosten MJ, Bressan RA, Li X. (2013) The Salt Overly Sensitive (SOS) pathway: established and emerging roles. *Mol Plant.* 6: 275-286.

Jiang M, Zhang J. (2002) Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. *J Exp Bot.* 53: 2401-2410.

Jiang, M, Zhang J. (2004) Absciscic acid and antioxidant defense in plant cells. *Acta Bot Sin.* 46: 1-9.

Jiménez A, Hernandez JA, Del Rio LA, Sevilla F. (1997) Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiol.* 114: 275-284.

Julkowska MM, Testerink C. (2015) Tuning plant signaling and growth to survive salt. *Trends Plant Sci.* 20: 586-594.

Kandar R, Kova P. (2008) Allantoin as a marker of oxidative stress in human erythrocytes. *Clin Chem Lab Med.* 46: 1270-1274.

Kandar R. (2016) The ratio of oxidized and reduced forms of selected antioxidants as a possible marker of oxidative stress in humans. *Biomed Chromatogr.* 30: 13-28.

Kaneyasu T, Kobayashi A, Nakayama M, Fujii N, Takahashi H, Miyazawa Y. (2007) Auxin response, but not its polar transport, plays a role in hydrotropism of Arabidopsis roots. *J Exp Bot.* 58: 1143-1150.

Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM. (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. *Plant Cell.* 9: 627-640.

Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G, Mullineaux P. (1999) Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. *Science.* 284: 654-657.



Karuppanapandian T, Moon JC, Kim C, Manoharan K, Kim K. (2011) Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. *Aust J Crop Sci.* 5: 709-725.

Khedr AH, Abbas MA, Wahid AA, Quick WP, Abogadallah GM. (2003) Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancreaticum maritimum* L. to salt-stress. *J Exp Bot.* 54: 2553-2562.

Kim K, Park J, Rhee S. (2007) Structural and functional basis for (S)-allantoin formation in the ureide pathway. *J Biol Chem.* 282: 23457-23464.

King CA, Purcell LC. (2005) Inhibition of N<sub>2</sub> fixation in soybean is associated with elevated ureides and amino acids. *Plant Physiol.* 137: 1389-1396.

Kliebenstein DJ, Monde RA, Last RL. (1998) Last Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. *Plant Physiol.* 118: 637-650.

Kohli A, Sreenivasulu N, Lakshmanan P, Kumar PP. (2013) The phytohormone crosstalk paradigm takes center stage in understanding how plants respond to abiotic stresses. *Plant Cell Rep.* 32: 945-957.

Koressaar T, Remm M. (2007) Enhancements and modifications of primer design program Primer3. *Bioinformatics.* 23: 1289-1291.

Komatsu S, Konishi H, Hashimoto M. (2007) The proteomics of plant cell membranes. *J Exp Bot.* 58: 103-112.

Kouchi H, Higuchi T. (1988) Carbon flow from nodulated roots to the shoots of soybean (*Glycine max* L. Men.) plant: An estimation of the contribution of current photosynthate to ureides in the xylem stream. *J Exp Bot.* 39: 1015-1023.

Krasensky J, Jonak C. (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *J Exp Bot.* 63: 1593-1608.

Kudoyarova GR, Vysotskaya LB, Cherkozyanova A, Dodd IC. (2007) Effect of partial rootzone drying on the concentration of zeatin-type cytokinins in tomato (*Solanum lycopersicum* L.) xylem sap and leaves. *J Exp Bot.* 58: 161-168.

- Ladrera R, Marino D, Larrainzar E, Gonzalez EM, ArreseIgor C. (2007) Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. *Plant Physiol.* 145: 539-546.
- Lake JA, Woodward FI, Quick WP. (2002) Long-distance CO<sub>2</sub> signalling in plants. *J Exp Bot.* 53: 183-193.
- Lamberto I, Percudani R, Gatti R, Folli C, Petrucco S. (2010) Conserved alternative splicing of Arabidopsis transthyretin-like determines protein localization and S-allantoin synthesis in peroxisomes. *Plant Cell.* 22: 1564-1574.
- Lange H, Shropshire W Jr, Mohr H. (1970) An analysis of phytochrome-mediated anthocyanin synthesis. *Plant Physiol.* 47: 649-655.
- Larson RA. (1988) The antioxidants of higher plants. *Phytochem.* 27: 969-978.
- Lawlor DW, Tezara W. (2009) Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: A critical evaluation of mechanisms and integration of processes. *Ann Bot.* 103: 561-579.
- Lee KH, Piao HL, Kim HY, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee IJ, Hwang I. (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell.* 126: 1109-1120.
- Legraverend M, Grierson DS. (2006) The purines: Potent and versatile small molecule inhibitors and modulators of key biological targets. *Bioorg Med Chem.* 14: 3987-3906.
- Lescano CI, Martini C, González CA, Desimone M. (2016) Allantoin accumulation mediated by allantoinase downregulation and transport by Ureide Permease 5 confers salt stress tolerance to Arabidopsis plants. *Plant Mol Biol.* 91: 581-95.
- Leung J, Giraudat J. (1998) Abscisic acid signal transduction. *Annu Rev Plant Physiol.* 49: 199-222.
- Li J, Qin RY, Li H, Xu RF, Yang YC, Ni DH, Ma H, Li L, Wei PC, Yang JB. (2015) Low-temperature-induced expression of rice ureidoglycolate amidohydrolase is mediated by a C-repeat/dehydration-responsive element that specifically interacts with rice C-repeat-binding factor 3. *Front Plant Sci.* 6: 1011-1018.

- Lichtenthaler HK, Buschmann C, Döll M, Fietz HJ, Bach T, Kozel U, Meier D, Rahmsdorf U. (1981). Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. *Photosynth Res.* 2: 115-141.
- Lichtenthaler HK, Wellburn AR. (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem Soc Trans.* 11: 591-592.
- Li TSC, Mazza G, Cottrell AC, Gao L. (1996) Ginsenosides in roots and leaves of american ginseng. *J Agric Food Chem.* 44: 717-720.
- Lim CW, Baek W, Jung J, Kim JH, Lee SC. (2015) Function of ABA in stomatal defense against biotic and drought stresses. *Int J Mol Sci.* 16: 15251-15270.
- Liu J, Zhu JK. (1998) A calcium sensor homolog required for plant salt tolerance. *Science.* 280: 1943-1945.
- Liu JH, Kitashiba H, Wang J, Ban Y, Moriguchi T. (2007) Polyamines and their ability to provide environmental stress tolerance to plants. *Plant Biotechnol.* 24: 117-126.
- Liu JX, Srivastava R, Che P, Howell SH. (2007) Salt stress responses in Arabidopsis utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. *Plant J.* 51: 897-909.
- Liu JH, Peng T, Dai WS. (2014) Critical *cis*-acting elements and interacting transcription factors: Key players associated with abiotic stress responses in plants. *Plant Mol Biol Rep.* 32: 303-317.
- Liu J-H, Wang W, Wu H, Gong X, Moriguchi T. (2015) Polyamines function in stress tolerance: from synthesis to regulation. *Front Plant Sci.* 6: 827-832.
- Livak KJ. (2001) ABI Prism 7700 Sequence Detection System. User bulletin 2, PE Applied Biosystems.
- Livak KJ, Schmittgen TD. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 25: 402-408.
- Mahajan S, Tuteja N. (2005) Cold, salinity and drought stresses: an overview. *Arch Biochem Biophys.* 444: 139-158.

- Malik VM, Lobo JM, Stewart C, Irani S, Todd CD, Gray GR. (2016) Growth irradiance affects ureide accumulation and tolerance to photoinhibition in *Eutrema salsugineum* (*Thellungiella salsuginea*). *Photosynthetica*. 54: 93-100.
- Mansfield TJ, Atkinson CJ. (1990). Stomatal behaviour in water stressed plants R.G. Alscher, J.R. Cumming (Eds.), Stress responses in plants: Adaptation and acclimation mechanisms, Wiley-Liss, New York. 241-264.
- Mattioli R, Falasca G, Sabatini S, Altamura MM, Costantino P, Trovato M. (2009), The proline biosynthetic genes *P5CS1* and *P5CS2* play overlapping roles in Arabidopsis flower transition but not in embryo development. *Physiol Plant*. 137: 72-85.
- Matsumoto T, Yamamoto Y, Yatazawa M. (1975) Role of root nodules in the nitrogen nutrition of soybeans. I. Fluctuation of allantoin and some other plant constituents in the growing period. *J Sci Soil Manure*. 46: 471-477.
- Mazzafera P, Goncalves, KV, Shimizu, MM. (2008) Control of allantoin accumulation in comfrey. *Nat Prod Commun*. 3: 1411-1422.
- Meot-Duros L, Magne C. (2009) Antioxidant activity and phenol content of *Crithmum maritimum* L. leaves. *Plant Physiol Biochem*. 47: 37-41.
- Mhamdi A, Queval G, Chaouch S, Vanderauwera S, Van Breusegem F, Noctor G. (2010) Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. *J Exp Bot*. 61: 4197-4220.
- Michalak A. (2006) Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. *Pol J Environ Stud*. 15: 523-530.
- Mishra Y, Johansson Jankapaa H, Kiss AZ, Funk C, Schroder WP, Jansson S. (2012) Arabidopsis plants grown in the field and climate chambers significantly differ in leaf morphology and photosystem components. *BMC Plant Biol*. 12: 6-14.
- Mitra J. (2001) Genetics and genetic improvement of drought resistance in crop plants. *Curr Sci*. 80: 758-763.
- Mittler R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*. 7: 405-510.

- Molassiotis A, Fotopoulos V. (2011) Oxidative and nitrosative signaling in plants two branches in the same tree? *Plant Signal Behav.* 6: 210-214.
- Moller IM, Jensen PE, Hansson A. (2007) Oxidative modifications to cellular components in plants. *Annu Rev Plant Biol.* 58: 459-481.
- Montalbini P. (1992) Ureides and enzymes of ureide synthesis in wheat seeds and leaves and effect of allopurinol on *Puccinia recondite* f. sp. *tritici* infection. *Plant Sci.* 87: 225-231.
- Montalbini P. (1995) Effect of rust infection on purine catabolism enzyme levels in wheat leaves. *Physiol Mol Plant Pathol.* 46: 275-292.
- Moons A, Prinsen E, Bauw G, Van Montagu M. (1997). Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. *Plant Cell.* 9: 2243-2259.
- Mothes K, Engelbrecht L. (1952) Uber allantoinsaure und allantoin. I. Ihre rolle als wanderform des stickstoffj und ihre beziehungen zum eiweibstoffwechsel des Aahorns. *Flora.* 139: 586-616.
- Mothes K, Engelbrecht L. (1954) Uber allantoinsaure und allantoin. II. Ihr vcrhalten in den speicherwurzeln von symphytum officinalt. *Flora.* 141: 356-378.
- Mothes K. (1961) The metabolism of urea and ureides. *Can J Bot.* 39: 1785-1807.
- Mullineaux P, Karpinski S. (2002) Signal transduction in response to excess light: getting out of the chloroplast. *Curr Opin Plant Biol.* 5: 43-48.
- Mullineaux P, Ball L, Escobar C, Karpinska B, Creissen G, Karpinski S. (2000) Are diverse signalling pathways integrated in the regulation of arabidopsis antioxidant defence gene expression in response to excess excitation energy? *Phil. Trans. R. Soc. B.* 355: 1531-1540.
- Murashige T, Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant.* 15: 473-497.
- Muthukumarasamy M, Gupta SD, Pannerselvam R. (2000) Enhancement of peroxidase, polyphenol oxidase and superoxide dismutase activities by tridimefon in NaCl-stressed *Raphanus sativus* L. *Biol Plant.* 43: 317-320.

- Mutlu F, Bozcuk S. (2007) Salinity-induced changes of free and bound polyamine levels in sunflower (*Helianthus annuus* L.) roots differing in salt tolerance. *Pak J Bot.* 39: 1097-1102.
- Myouga F, Hosoda C, Umezawa T, Iizumi H, Kuromori T, Motohashi R, Shono Y, Nagata N, Ikeuchi M, Shinozaki K. (2008) A heterocomplex of iron superoxide dismutases defends chloroplast nucleoids against oxidative stress and is essential for chloroplast development in Arabidopsis. *Plant Cell.* 20: 3148-3162.
- Nakabayashi R, Saito K. (2015) Integrated metabolomics for abiotic stress responses in plants. *Curr Opin Plant Biol.* 24: 10-6.
- Nakagawa A, Sakamoto S, Takahashi M, Morikawa H, Sakamoto A. (2007) The RNAi-mediated silencing of xanthine dehydrogenase impairs growth and fertility and accelerates leaf senescence in transgenic Arabidopsis plants. *Plant Cell Physiol.* 48: 1484-1495.
- Nam MH, Bang E, Kwon TY, Kim Y, Kim EH, Cho K, Park WJ, Kim BG, Yoon IS. (2015) Metabolite profiling of diverse rice germplasm and identification of conserved metabolic markers of rice roots in response to long-term mild salinity stress. *Int J Mol Sci.* 16: 21959-21974.
- Nambara E, A Marion-Poll. (2005) Absciscic Acid Biosynthesis and Catabolism. *Annu Rev Plant Biol.* 56: 165-185.
- Nasmyth KA, Reed SI. (1980) Isolation of genes by complementation in yeast: molecular cloning of a cell-cycle gene. *Proc. Natl Acad Sci U S A.* 77: 2119-2123.
- Natella F, Maldini M, Leoni G, Scaccini C. (2014) Glucosinolates redox activities: can they act as antioxidants? *Food Chem.* 149: 226-232.
- Navarro JM, Flores P, Garrido C, Martinez V. (2006) Changes in the contents of antioxidant compounds in pepper fruits at ripening stages, as affected by salinity. *Food Chem.* 96: 66-73.
- Neves-Borges AC, Guimarães-Dias F, Cruz F, Mesquita RO, Nepomuceno AL, Romano E, Loureiro ME, de Fátima Grossi-de-Sá M, Alves-Ferreira M. (2012) Expression pattern of drought stress marker genes in soybean roots under two water deficit systems. *Genet Mol Biol.* 35: 212-221.
- Nishizawa A, Yabuta Y, Shigeoka S. (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol.* 147: 1251-1263.

- Noh B, Spalding EP. (1998) Anion channels and the stimulation of anthocyanin accumulation by blue light in *Arabidopsis* seedlings. *Plant Physiol.* 116: 503-509.
- Nounjan N, Nghia PT, Theerakulpisut P. (2012) Exogenous proline and trehalose promote recovery of rice seedlings from salt-stress and differentially modulate antioxidant enzymes and expression of related genes. *J Plant Physiol.* 169: 596-604.
- Nouri MZ, Moumeni A, Komatsu S. (2015) Abiotic stresses: Insight into gene regulation and protein expression in photosynthetic pathways of plants. *Int J Mol Sci.* 16: 20392-20416.
- Ogawa K, Kanematsu S, Tabake K, Asada K. (1995) Attachment of CuZn-superoxide dismutase to thylakoid membranes at the site of superoxide generation (PSI) in spinach chloroplasts: detection by immuno-gold labeling after rapid freezing and substitution method. *Plant Cell Physiol.* 36: 565-573.
- Okubo K, Yoshiki Y. (2000) The role of triterpenoid on reactive oxygen scavenging system: Approach from the new chemiluminescence system (XYZ system). *Biofactors.* 13: 219-223.
- Oliver MJ, Guo L, Alexander DC, Ryals JA, Wone BW, Cushman JC. (2011) A sister group contrast using untargeted global metabolomic analysis delineates the biochemical regulation underlying desiccation tolerance in *Sporobolus stapfianus*. *Plant Cell.* 23: 1231-1248.
- Ondrasek G, Rengel Z, Veres S. (2011) Soil salinisation and salt stress in crop production. In: Shanker AK, Venkateswarlu B. eds. Abiotic stress in plants: mechanisms and adaptations. Rijeka, Croatia: InTech. pp 171-190.
- Oono Y, Seki M, Nanjo T, Narusaka M, Fujita M, Satoh R, Satou M, Sakurai T, Ishida J, Akiyama K, Iida K, Maruyama K, Satoh S, Yamaguchi-Shinozaki K, Shinozaki K. (2003) Monitoring expression profiles of *Arabidopsis* gene expression during rehydration process after dehydration using ca 7000 full-length cDNA microarray. *Plant J.* 34: 868-887.
- Pant BD, Pant P, Erban A, Huhman D, Kopka J, Scheible WR. (2015) Identification of primary and secondary metabolites with phosphorus status-dependent abundance in *Arabidopsis*, and of the transcription factor PHR1 as a major regulator of metabolic changes during phosphorus limitation. *Plant Cell Environ.* 38: 172-187.

Parida AK, Das AB. (2005) Salt tolerance and salinity effects on plants: A review. *Ecotoxicol Environ Saf.* 60: 324-349.

Parihar P, Singh S, Singh R, Singh VP, Prasad SM. (2015) Effect of salinity stress on plants and its tolerance strategies: A review. *Environ Sci Pollut Res Int.* 22: 4056-4075.

Parker J. (1968). Drought-resistance mechanisms. In *Water Deficits and Plant Growth*. T.T. Kozlowsky, ed. New York: Academic Press. pp 195-234.

Passioura J. (1997) Drought and drought tolerance. *Drought tolerance in higher plants: Genetical, physiological and molecular biological analysis*. Netherlands: Springer. pp 1-5.

Pastori GM, Del Rio LA. (1997) Natural senescence of pea leaves: An activated oxygen-mediated function for peroxisomes. *Plant Physiol.* 113: 411-418.

Peleg Z, Blumwald E. (2011) Hormone balance and abiotic stress tolerance in crop plants. *Curr Opin Plant Biol.* 14: 290-295.

Pelissier HC, Frerich A, Desimone M, Schumacher K, Tegeder M. (2004) PvUPS1, an allantoin transporter in nodulated roots of French bean. *Plant Physiol.* 134: 664-675.

Peoples MB, Pate JS, Atkins CA. (1985) The effect of nitrogen source on transport and metabolism of nitrogen in fruiting plants of cowpea (*Vigna unguiculata* (L.) Walp.). *J Exp Bot.* 36: 567-582.

Pessoa J, Sárkány Z, Ferreira-da-Silva F, Martins S, Almeida MR, Li J, Damas AM. (2010) Functional characterization of *Arabidopsis thaliana* transthyretin-like protein. *BMC Plant Biol.* 10: 30-36.

Perry JJ, Shin DS, Getzoff ED, Tainer JA. (2010) The structural biochemistry of the superoxide dismutases. *Biochim Biophys Acta.* 1804: 245-262.

Petrov VD, Van Breusegem F. (2012) Hydrogen peroxide-a central hub for information flow in plant cells. *AoB Plants.* 2012: pls014.

Petrov V, Hille J, Mueller-Roeber B, Gechev TS. (2015) ROS-mediated abiotic stress-induced programmed cell death in plants. *Front Plant Sci.* 6: 69-76.



- Petrusa LM, Winicov I. (1997) Proline status in salt tolerant and salt sensitive alfalfa cell lines and plants in response to NaCl. *Plant Physiol Biochem.* 35: 303-310.
- Pfannschmidt T, Nilsson A, Allen JF. (1999) Photosynthetic control of chloroplast gene expression. *Nature.* 397: 625-628.
- Pierik R, Tholen D, Poorter H, Visser EJ, Voeseek LA. (2006) The Janus face of ethylene: Growth inhibition and stimulation. *Trends Plant Sci.* 11:176-183.
- Pinheiro C, Chaves MM. (2011) Photosynthesis and drought: Can we make metabolic connections from available data? *J Exp Bot.* 62: 869-882.
- Pintó-Marijuan M, Munné-Bosch S. (2014) Photo-oxidative stress markers as a measure of abiotic stress-induced leaf senescence: Advantages and limitations. *J Exp Bot.* 65: 3845-3857.
- Pilon M, Ravet K, Tapken W. (2011) The biogenesis and physiological function of chloroplast superoxide dismutases. *Biochim Biophys Acta.* 1807: 989-998.
- Pompelli MF, Pompelli GM, de Oliveira AFM, Antunes WC. (2013) The effect of light and nitrogen availability on the caffeine, theophylline and allantoin contents in the leaves of *Coffea arabica* L. *AIMS Environ Sci.* 1: 1-11.
- Pope FG. (1919) Modern research in organic chemistry. Second Edition. New York: D. Van Nostrand Co.
- Price AH, Cairns JE, Horton P, Jones HG, Griffiths H. (2002) Linking drought-resistance mechanisms to drought avoidance in upland rice using a QTL approach: progress and new opportunities to integrate stomatal and mesophyll responses. *J Exp Bot.* 53: 989-1004.
- Proctor P. (1970) Similar functions of uric acid and ascorbate in man? *Nature.* 228: 868-875.
- Pospisilova J, Vagner M, Malbeck J, Travniakova A, Batkova P. (2005) Interactions between abscisic acid and cytokinins during water stress and subsequent rehydration. *Biol Plant.* 49: 533-540.
- Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene.* 111: 229-233.
- Prelich G. (2012) Gene overexpression: uses, mechanisms, and interpretation. *Genetics.* 190: 841-854.

- Prestridge DS. (1995) Predicting pol II promoter sequences using transcription factor binding sites. *J Mol Biol.* 249: 923-932.
- Puntarulo S, Sánchez RA, Boveris A. (1988) Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. *Plant Physiol.* 86: 626-630.
- Purcell LC, Serraj R, De Silva M, Sinclair TR, Bona S. (1998) Ureide concentration in field-grown soybean in response to drought and the relationship to nitrogen fixation. *J Plant Nutr.* 21: 949-966.
- Purcell LC, King CA, Ball RA. (2000) Soybean cultivar differences in ureides and the relationship to drought tolerant nitrogen fixation and manganese nutrition. *Crop Sci.* 40: 1062-1070.
- Qin F, Shinozaki K, Yamaguchi-Shinozaki K. (2011) Achievements and challenges in understanding plant abiotic stress responses and tolerance. *Plant Cell Physiol.* 52: 1569-1582.
- Quan R, Lin H, Mendoza I, Zhang Y, Cao W, Yang Y, Shang M, Chen S, Pardo M, Guo Y. (2007) SCABP8/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress. *Plant Cell.* 19: 1415-1431.
- Radin JW, Parker LL, Guinn G. (1982) Water relations of cotton plants under nitrogen deficiency. V. Environmental control of abscisic acid accumulation and stomatal sensitivity to abscisic acid. *Plant Physiol.* 70: 1066-1070.
- Rajala A, Peltonen-Sainio P. (2001) Plant growth regulator effects on spring cereal root and shoot growth. *Agron J.* 93: 936-943.
- Ramakrishna A, Dayananda C, Giridhar P, Rajasekaran T, Ravishankar GA. (2011) Photoperiod influences endogenous indoleamines in cultured green alga *Dunaliella bardawil*. *Indian J Exp Biol.* 49: 234-240.
- Ramakrishna A, Ravishankar GA. (2011) Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal Behav.* 6:1720-1731.
- Ramazzina I, Folli C, Secchi A, Berni R, Percudani R. (2006) Completing the uric acid degradation pathway through phylogenetic comparison of whole genomes. *Nat Chem Biol.* 2: 144-148.

Ramel F, Sulmon C, Bogard M, Couée I, Gouesbet G. (2009) Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. *BMC Plant Biol.* 9: 28-35.

Rawsthorne S, Minchin FR, Summerfield RJ, Cockson C, Coombs J. (1980) Carbon and nitrogen metabolism in legume root nodules. *Phytochem.* 19: 341-355.

Reddy AR, Raghavendra AS. (2006) Photooxidative stress. Physiology and molecular biology of stress tolerance in plants. Netherlands: Springer. pp 157-186.

Reinbothe H, Mothes K. (1962) Urea, ureides, and guanidines in plants. *Ann Rev Plant Physiol.* 13: 129-150.

Reumann S, Babujee L, Ma C, Wienkoop S, Siemsen T, Antonicelli GE, Rasche N, Lüder F, Weckwerth W, Jahn O. (2007) Proteome analysis of Arabidopsis leaf peroxisomes reveals novel targeting peptides, metabolic pathways, and defense mechanisms. *Plant Cell.* 19: 3170-3193.

Rhodes D, Nadolska-Orczyk A, Rich PJ. (2002) Salinity, osmolytes and compatible solutes. Salinity: environment-plants-molecules. Netherland: Kluwer Academic. pp 181-204.

Rodriguez AA, Grunberg KA, Taleisnik EL. (2002) Reactive oxygen species in the elongation zone of maize leaves are necessary for leaf extension. *Plant Physiol.* 129: 1627-1632.

Rosemeyer H. (2004) The chemodiversity of purine as a constituent of natural products. *Chem Biodivers.* 1: 361-301.

Ruban AV. (2009) Plants in light. *Commun Integr Biol.* 2: 50-55.

Sambrook J, Russell DW. (2001) Molecular Cloning: A Laboratory Manual. Third Edition. New York: Cold Spring Harbor Laboratory Press.

Sautin YY, Johnson RJ. (2008) Uric acid: the oxidant-antioxidant paradox. *Nucleos Nucleot Nucl.* 27: 608-619.

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, Xu D, Hellsten U, May GD, Yu Y, Sakurai T, Umezawa T, Bhattacharyya MK, Sandhu D, Valliyodan B, Lindquist E, Peto M, Grant D, Shu S, Goodstein D, Barry K, Futrell-Griggs M, Abernathy B, Du J, Tian Z,

- Zhu L, Gill N, Joshi T, Libault M, Sethuraman A, Zhang XC, Shinozaki K, Nguyen HT, Wing RA, Cregan P, Specht J, Grimwood J, Rokhsar D, Stacey G, Shoemaker RC, Jackson SA. (2010) Genome sequence of the palaeopolyploid soybean. *Nature*. 463: 178-183.
- Schneider CA, Rasband WS, Eliceiri KW. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 9: 671-675.
- Schoelz JE, Bourque JE. (1999) Plant pararetroviruses (Caulimoviridae). In: Granoff A, Webster RB, eds. *Encyclopedia of Virology*. San Diego: Academic Press. pp 1275-1281.
- Schubert KR. (1986) Products of biological nitrogen fixation in higher plants: Synthesis, transport and metabolism. *Annu Rev Plant Physiol*. 37: 539-574.
- Schuppler U, He PH, John PCL, Munns R. (1998) Effects of water stress on cell division and cell-division-cycle-2-like cell-cycle kinase activity in wheat leaves. *Plant Physiol*. 117: 667-678.
- Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K. (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold, and high-salinity stresses using a full-length cDNA microarray. *Plant J*. 31: 279-292.
- Serraj R, Sinclair TR. (1996) Processes contributing to N<sub>2</sub>-fixation insensitivity to drought in the soybean cultivar Jackson. *Crop Sci*. 36: 961-968.
- Serraj R, Vadez VV, Denison RF, Sinclair TR. (1999) Involvement of ureides in nitrogen fixation inhibition in soybean. *Plant Physiol*. 119: 289-296.
- Serraj R, Vadez V, Sinclair TR. (2001) Feedback regulation of symbiotic N<sub>2</sub> fixation under drought stress. *Agronomie*. 21: 621-626.
- Serventi F, Ramazzina I, Lamberto I, Puggioni V, Gatti R, Percudani R. (2010) Chemical basis of nitrogen recovery through the ureide pathway: Formation and hydrolysis of S-ureidoglycine in plants and bacteria. *ACS Chem Biol*. 5: 203-214.
- Shahidi F, Chandrasekara A. (2010) Hydroxycinnamates and their *in vitro* and *in vivo* antioxidant activities. *Phytochem Rev*. 9: 147-170.

Shao H, Chu L, Lu ZH, Kang CM. (2008) Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. *Int J Biol Sci.* 4: 8-14.

Sharma P, Jha AB, Dubey RS, Pessarakli M. (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J Bot.* 2012: 1-12.

Sharp RE. (2002) Interaction with ethylene: Changing views on the role of abscisic acid in root and shoot growth responses to water stress. *Plant Cell Environ.* 25: 211-222.

Shavrukov Y. (2013) Salt stress or salt shock: Which genes are we studying? *J Exp Bot.* 64: 119-127.

Shelp BJ, Atkins CA, Storer PJ, Canvin DT. (1983) Cellular and subcellular organization of pathways of ammonia assimilation and ureide synthesis in nodules of cowpea (*Vigna unguiculata* L. Walp.). *Arch Biochem Biophys.* 224: 429-441.

Shelp BJ, Ireland RJ. (1985) Ureide metabolism in leaves of nitrogen-fixing soybean plants. *Plant Physiol.* 77: 779-83.

Shelp BJ, Da Silva MC. (1990) Distribution and metabolism of xylem-borne ureido and amino compounds in developing soybean shoots. *Plant Physiol.* 94: 1505-1511.

Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura K. (2002) Regulation and function of ascorbate peroxidase isoenzymes. *J Exp Bot.* 53: 1305-1319.

Shinozaki K, Yamaguchi-Shinozaki K. (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr Opin Plant Biol.* 3: 217-223.

Shinozaki K, Yamaguchi-Shinozaki K. (2007). Gene networks involved in drought stress response and tolerance. *J Exp Bot.* 58: 221-227.

Signorelli S, Coitiño EL, Borsani O, Monza J. (2014) Molecular mechanisms for the reaction between (.)OH radicals and proline: Insights on the role as reactive oxygen species scavenger in plant stress. *J Phys Chem B.* 118: 37-47.

Sinclair TR, Serraj R. (1995) Legume nitrogen fixation and drought. *Nature.* 378: 344-347.

Singh R, Jwa NS. (2013) The rice MAPKK-MAPK interactome: the biological significance of MAPK components in hormone signal transduction. *Plant Cell Rep.* 32: 923-931.

Smillie RM, Hetherington SE. (1999) Photoabatement by anthocyanin shields photosynthetic systems from light stress. *Photosynthetica.* 36: 451-463.

Smirnoff N, Cumbes QJ. (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochem.* 28: 1057-1060.

Sobahan MA, Arias CR, Okuma E, Shimoishi Y, Nakamura Y, Hirai Y, Mori IC, Murata Y. (2009) Exogenous proline and glycinebetaine suppress apoplastic flow to reduce Na(+) uptake in rice seedlings. *Biosci Biotechnol Biochem.* 73: 2037-2042.

Soliz-Guerrero JB, de Rodriguez DJ, RodriguezGarcia R, Angulo-Sanchez JL, Mendez-Padilla G. (2002) Quinoa saponins: Concentration and composition analysis. In: Janick J, Whipkey A, Eds. Trends in new crops and new uses. Alexandria: ASHS Press: pp 110-114.

Solovyev VV, Shahmuradov IA. (2003) PromH: Promoters identification using orthologous genomic sequences. *Nucleic Acids Res.* 31: 3540-3545.

Sousa MP, Matos MEO, Matos FJA, Machado MIL, Craveiro AA. (1991) Constituintes químicos ativos de plantas medicinais brasileiras. Editora da Universidade Federal do Ceará, Fortaleza, Brazil.

Springer P. (2000) Gene traps: Tools for plant development and genomics. *Plant Cell.* 12: 1007-1020.

Stasolla C, Katahira R, Thorpe TA, Ashihara H. (2003) Purine and pyrimidine nucleotide metabolism in higher plants. *J Plant Physiol.* 160: 1271-1295.

Steyn WJ, Wand SJE, Holcroft DM, Jacobs G. (2002). Anthocyanins in vegetative tissues: Anthocyanins in unified function a proposed photoprotection. *New Phytol.* 155: 349-361.

Streeter JG. (1992) Transport and metabolism of carbon and nitrogen in legume nodules. *Adv Bot Res.* 18: 130-187.

Szabados L, Savoure A. (2010) Proline: a multifunctional amino acid. *Trends Plant Sci.* 15: 89-97.

- Szabados L, Kovacs H, Zilberstein A, Bouchereau A. (2011) Plants in extreme environments: Importance of protective compounds in stress tolerance. *Adv Bot Res.* 57: 105-150.
- Tajima S, Nomura M, Kouchi H. (2004) Ureide biosynthesis in legume nodules. *Front Biosci.* 9: 1374-1381.
- Takagi H, Ishiga Y, Watanabe S, Konishi T, Egusa M, Akiyoshi N, Matsuura T, Mori IC, Hirayama T, Kaminaka H, Shimada H, Sakamoto A. (2016) Allantoin, a stress-related purine metabolite, can activate jasmonate signaling in a MYC2-regulated and abscisic acid-dependent manner. *J Exp Bot.* 67: 2519-2532.
- Tani T, Sobajima H, Okada K, Chujo T, Arimura S, Tsutsumi N, Nishimura M, Seto H, Nojiri H, Yamane H. (2008). Identification of the *OsOPR7* gene encoding 12-oxophytodienoate reductase involved in the biosynthesis of jasmonic acid in rice. *Planta.* 227: 517-526.
- Tavakkoli E, Fatehi F, Coventry S, Rengasamy P, McDonald GK. (2011) Additive effects of Na<sup>+</sup> and Cl<sup>-</sup> ions on barley growth under salinity stress. *J Exp Bot.* 62: 2189-2203.
- Taylor NL, Tan YF, Jacoby RP, Millar AH. (2009) Abiotic environmental stress induced changes in the *Arabidopsis thaliana* chloroplast, mitochondria and peroxisome proteomes. *J Proteomics.* 72: 367-378.
- Tegeder M. (2014) Transporters involved in source to sink partitioning of amino acids and ureides: opportunities for crop improvement. *J Exp Bot.* 65: 1865-1878.
- Thomas RJ, Schrader LE. (1981) Ureide metabolism in higher plants. *Phytochem.* 20: 361-371.
- Thomashow MF. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanism. *Annu. Rev. Plant Physiol.* 50: 571-599.
- Thomashow MF. (2010) Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiol.* 154: 571-577.
- Tiburcio AF, Altabella T, Bitrián M, Malcázar R. (2014) The roles of polyamines during the lifespan of plants: from development to stress. *Planta.* 240: 1-18.
- Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S. (2002) Agricultural sustainability and intensive production practices. *Nature.* 418: 671-677.

- Todd CD, Tipton PA, Blevins DG, Piedras P, Pineda M, Polacco JC. (2006) Update on ureide degradation in legumes. *J Exp Bot.* 57: 5-12.
- Todd CD, Polacco JC. (2006) AtAAH encodes a protein with allantoate amidohydrolase activity from *Arabidopsis thaliana*. *Planta.* 223: 1108-1113.
- Tracey MV. (1955) Urea and ureides. In: Peach K, Tracey MV, eds. Modern methods of plant analysis. Vol. IV. Berlin: Springer-Verlag. pp 119-141.
- Triplett EW, Blevins DG, Randall DD. (1982) Purification and properties of soybean nodule xanthine dehydrogenase. *Arch Biochem. Biophys.* 219: 39-46.
- Tuteja N. (2007) Absciscic acid and abiotic stress signaling. *Plant Signal Behav.* 2: 135-138.
- Tuteja N, Sopory SK. (2008) Chemical signaling under abiotic stress environment in plants. *Plant Signal Behav.* 2: 525-536.
- Untergrasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. (2012) Primer3 - new capabilities and interfaces. *Nucleic Acids Res.* 40: e115- e122.
- Upadhyaya H, Sahoo L, Panda SK. (2013) Molecular physiology of osmotic stress in plants. In: Molecular stress physiology of plants. New York: Springer. pp 179-192.
- Vadez V, Sinclair TR, Serraj R. (2000) Asparagine and ureide accumulation in nodules and shoots as feedback inhibitors of N<sub>2</sub> fixation in soybean. *Physiol Plant.* 110: 215-223.
- Valluru R, Van den Ende W. (2008) Plant fructans in stress environments: emerging concepts and future prospects. *J Exp Bot.* 59: 2905-2916.
- Velikova VB, Edreva AM, Tsonev TD, Jones HG. (2007) Singlet oxygen quenching by phenylamides and their parent compounds. *Z Naturforsch C.* 62: 833-838.
- Ventura Y, Wuddineh WA, Ephrath Y, Shpigel M, Sagi M. (2010) Molybdenum as an essential element for improving total yield in seawater-grown *Salicornia europaea* L. *Sci Hort.* 126: 395-401.



- Ventura Y, Myrzabayeva M, Alikulov Z, Omarov R, Khozin-Goldberg I, Sagi M. (2014) Effects of salinity on flowering, morphology, biomass accumulation and leaf metabolites in an edible halophyte. *AoB Plants*. 6: plu053.
- Verbruggen N, Hermans C. (2008) Proline accumulation in plants: A review. *Amino Acids*. 35: 753-759.
- Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK. (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J*. 45: 523-539.
- Vickers CE, Gershenzon J, Lerdau MT, Loreto F. (2009) A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nat Chem Biol*. 5: 283-291.
- Visscher AM, Belfield EJ, Vlad D, Irani N, Moore I, Harberd NP. (2015) Overexpressing the multiple-stress responsive gene At1g74450 reduces plant height and male fertility in *Arabidopsis thaliana*. *PLoS One*. 10: e0140368.
- Vitória AP, Mazzafera P. (1999) Xanthine degradation and related enzyme activities in leaves and fruits of two coffee species differing in caffeine catabolism. *J Agric Food Chem*. 47: 1851-1855.
- Vogelman TC, Nishio JN, Smith WK. (1996) Leaves and light capture: Light propagation and gradients of carbon fixation within leaves. *Trends Plant Sci*. 1: 65-70.
- Walker MA, Dumbroff EB. (1981) Effects of salt stress on abscisic-acid and cytokinin levels in tomato. *Z Pflanzenphysiol*. 101: 461-470.
- Wang DH, Du F, Liu HY, Liang ZS. (2010a) Drought stress increases iridoid glycosides biosynthesis in the roots of *Scrophularia ningpoensis* seedlings. *J Med Plants Res*. 4: 2691-2699.
- Wang P, Kong CH, Hu F, Xu XH. (2007) Allantoin involved in species interactions with rice and other organisms in paddy soil. *Plant Soil*. 296: 43-51.
- Wang P, Kong CH, Sun B, Xu XH. (2010b) Allantoin-induced change of microbial diversity and community in rice soil. *Plant Soil*. 332: 357-368.
- Wang P, Kong CH, Sun B, Xu XH. (2012) Distribution and function of allantoin (5-ureidohydantoin) in rice grains. *J Agric Food Chem*. 60: 2793-2798.

- Wang WS, Zhao XQ, li M, Huang LY, Xu JL, Zhang F, Cui YR, Fu BY, Li ZK. (2016) Complex molecular mechanisms underlying seedling salt tolerance in rice revealed by comparative transcriptome and metabolomic profiling. *J Exp Bot.* 67: 405-419.
- Wang Y, Li K, Li X. (2009) Auxin redistribution modulates plastic development of root system architecture under salt stress in *Arabidopsis thaliana*. *J Plant Physiol.* 166: 1637-1645.
- Watanabe S, Kounosu Y, Shimada H, Sakamoto A. (2014b) Arabidopsis xanthine dehydrogenase mutants defective in purine degradation show a compromised protective response to drought and oxidative stress. *Plant Biotechnol.* 31: 173-178.
- Watanabe S, Matsumoto M, Hakomori Y, Takagi H, Shimada H, Sakamoto A. (2014a) The purine metabolite allantoin enhances abiotic stress tolerance through synergistic activation of abscisic acid metabolism. *Plant Cell Environ.* 37: 1022-1036.
- Watanabe S, Nakagawa A, Izumi S, Shimada H, Sakamoto A. (2010) RNA interference-mediated suppression of xanthine dehydrogenase reveals the role of purine metabolism in drought tolerance in Arabidopsis. *FEBS Lett.* 584: 1181-1186.
- Weigel D, Glazebrook J. (2008) Genetic analysis of Arabidopsis mutants. *CSH Protoc.* 3: 1-6.
- Werner AK, Sparkes IA, Romeis T, Witte CP. (2008) Identification, biochemical characterization, and subcellular localization of allantoin amidohydrolases from Arabidopsis and soybean. *Plant Physiol.* 146: 418-430.
- Werner AK, Romeis T, Witte CP. (2010) Ureide catabolism in *Arabidopsis thaliana* and *Escherichia coli*. *Nat Chem Biol.* 6: 19-21.
- Werner AK, Witte CP. (2011) The biochemistry of nitrogen mobilization: Purine ring catabolism. *Trends Plant Sci.* 16: 381-387.
- Wilkinson S, Davies WJ. (2002) ABA-based chemical signalling: The co-ordination of responses to stress in plants. *Plant Cell Environ.* 25: 195-210.
- Wilkinson S, Kudoyarova GR, Veselov DS, Arkhipova TN, Davies WJ. (2012) Plant hormone interactions: innovative targets for crop breeding and management. *J Exp Bot.* 63: 3499-3509.

- Wituszyńska W, Karpiński S. (2013) Programmed cell death as the response to high light, UV and drought stress in plants, In: Vahdati K, Leslie Ch, Abiotic stress plant responses and applications in agriculture. In Tech. pp 207-231.
- Wu X, Muzny DM, Lee CC, Caskey CT. (1992) Two independent mutational events in the loss of urate oxidase during hominoid evolution. *J Mol Evol.* 34: 78-84.
- Xiong L, Zhu JK. (2002) Molecular and genetic aspects of plant responses to osmotic stress. *Plant Cell Environ.* 25: 131-139.
- Xu W, Jia L, Shi W, Liang J, Zhou F, Li Q, Zhang J. (2013) Absciscic acid accumulation modulates auxin transport in the root tip to enhance proton secretion for maintaining root growth under moderate water stress. *New Phytol.* 197: 139-150.
- Xu Z, Zhou G, Shimizu H. (2010) Plant responses to drought and rewatering. *Plant Signal Behav.* 5: 649-654.
- Yamaguchi-Shinozaki K, Koizumi M, Urao S, Shinozaki K. (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*, sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. *Plant Cell Physiol.* 33: 217-224.
- Yamaguchi-Shinozaki K, Shinozaki K. (1993) Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. *Mol Gen Genet.* 236: 331-340.
- Yamaguchi-Shinozaki K, Shinozaki K. (1994) A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell.* 6: 251-264.
- Yang F, Moss LC, Phillips GN. (1996) The molecular structure of green fluorescent protein. *Nat Biotechnol.* 14: 1246-1251.
- Yang J, Han KH. (2004) Functional characterization of allantoinase genes from *Arabidopsis* and a nonureide-type legume black locust. *Plant Physiol.* 134: 1039-1049.
- Yardim-Akaydin S, Sepici A, Ozkan Y, Simsek B, Sepici V. (2006) Evaluation of allantoin levels as a new marker of oxidative stress in Behcet's disease. *Scand J Rheumatol.* 35: 61-64.

- Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 13: 134-140.
- Yesbergenova Z, Yang G, Oron E, Soffer D, Fluhr R, Sagi M. (2005) The plant Mo-hydroxylases aldehyde oxidase and xanthine dehydrogenase have distinct reactive oxygen species signatures and are induced by drought and abscisic acid. *Plant J*. 42: 862-876.
- Yeum KJ, Russell RM, Krinsky NI, Aldini G. (2004) Biomarkers of antioxidant capacity in the hydrophilic and lipophilic compartments of human plasma. *Arch Biochem Biophys*. 430: 97-103.
- Yoshida T, Mogami J, Yamaguchi-Shinozaki K. (2014) ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Curr Opin Plant Biol*. 21: 133-139.
- Yobi A, Wone BW, Xu W, Alexander DC, Guo L, Ryals JA, Oliver MJ, Cushman JC. (2013) Metabolomic profiling in *Selaginella lepidophylla* at various hydration states provides new insights into the mechanistic basis of desiccation tolerance. *Mol Plant*. 6: 369-385.
- Zhang X, Henriques R, Lin SS, Niu QW, Chua NH. (2006) Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat Protoc*. 1: 641-646.
- Zhao Y, Wang T, Zhang W, Li X. (2011) SOS3 mediates lateral root development under low salt stress through regulation of auxin redistribution and maxima in *Arabidopsis*. *New Phytol*. 189: 1122-1134.
- Zhu JK. (2002) Salt and drought stress signal transduction in plants. *Annu Rev Plant Physiol*. 53: 247-273.
- Zolla G, Heimer YM, Barak S. (2010) Mild salinity stimulates a stress-induced morphogenic response in *Arabidopsis thaliana* roots. *J Exp Bot*. 61: 211-224.
- Zwack PJ, Rashotte AM. (2015) Interactions between cytokinin signalling and abiotic stress responses. *J Exp Bot*. 66: 4863-4871.